AD NUMBER

ADB285707

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies only; Proprietary Information; Jul 2002. Other requests shall be referred to USAMRMC, Ft. Detrick, MD 21702

AUTHORITY

USAMRMC ltr, dtd 28 July 2003

AD_____

Award Number: DAMD17-98-1-8272

TITLE: Mammalian Homologs of Yeast Checkpoint Genes

PRINCIPAL INVESTIGATOR: David F. Stern, Ph.D.

CONTRACTING ORGANIZATION: Yale University
New Haven, Connecticut 06520-8047

REPORT DATE: July 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

NOTICE

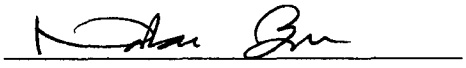
USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-98-1-8272
Organization: Yale University

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.



12/5/02

_____ _____

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Final (1 Jul 98 - 30 Jun 02)

4. TITLE AND SUBTITLE
Mammalian Homologs of Yeast Checkpoint Genes

5. FUNDING NUMBERS
DAMD17-98-1-8272

6. AUTHOR(S)
David F. Stern, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Yale University
New Haven, Connecticut 06520-8047

E-Mail:DF.Stern@yale.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Jul 02). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
We hypothesize that components of DNA-related checkpoint pathways in addition to members of the ATM protein/lipid kinase family are conserved in all eukaryotes. This is based on functional similarities in the pathways and the conservation between the evolutionarily disparate budding and fission yeasts. Work described herein discusses models for ATM-dependent regulation of the effector kinase Chk2 and other aspects of Chk2 regulation. The significance for DNA damage-dependent regulation of Chk2 is discussed.

14. SUBJECT TERMS
breast cancer, checkpoint control, saccharomyces cerevisiae, MEC1, Rad53, ATM

15. NUMBER OF PAGES
45

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

✓ Where copyrighted material is quoted, permission has been obtained to use such material.

✓ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

✓ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

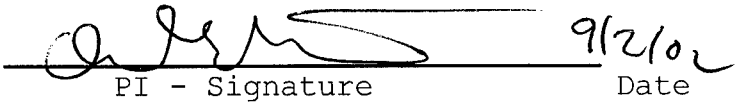
In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



PI - Signature 9/2/02 Date

5) INTRODUCTION

We originally hypothesized that components of DNA-related checkpoint pathways in addition to members of the *ATM* protein/lipid kinase family are conserved in all eukaryotes. This was based on functional similarities in the pathways and the conservation between the evolutionarily disparate budding and fission yeasts [1]. Our goal was to identify additional regulators of mammalian DNA checkpoints by virtue of structural and functional homology with known checkpoint genes in budding yeast. We had proposed to use both structural and functional screens to identify human homologs of yeast damage checkpoint proteins Rad53 and Rad9. Once identified, such components would be ordered into pathways for mammalian checkpoint function, with emphasis on p53 regulation, cell cycle regulation, and complementation of *ATM* defects. Experimental work over the past few years has completely confirmed this hypothesis, and led to major advances in understanding mammalian checkpoint systems. Our work was to identify a hypothetical mammalian homolog of Rad53 (Technical Objectives 1,2,3), and, when the homolog Chk2 was identified, to elucidate regulation of mammalian Rad53 (Chk2/Cds1), and regulation of its targets (Technical Objective 4).

(6) BODY

Year 1.

I.**Technical Objective 1.** The overall objective of Objective 1 was to use protein-based screens in order to identify mammalian homologs of yeast Rad53 and Rad9. In order to do this, the relevant protein interaction domains were to be used as baits in protein interaction screens for novel mammalian partners.

A.Tools for mapping interaction of Mec1 with Rad53 and Rad9. The first strategy was to identify a domain of (yeast) Mec1 that is responsible for interaction with Rad53 and/or Rad9, and then to use the homologous domain from (mammalian) Atm to identify dimerization partners. Since this aim first required that we express functional Mec1 in order to map interactions with Rad53, we endeavored to express this large gene in yeast and in mammalian cells. A *MEC1* gene was cloned from yeast by recombinational rescue, and moved into vectors for expression in mammalian cells, bacteria, and budding yeast. Efforts to express the polypeptide (marked with a V5 epitope tag) through transient transfection into COS-7 cells have thus far been unsuccessful. This is not completely surprising, since there is likely to be functional conservation of Mec1 and Atm, and Atm has proven to be difficult to express in mammalian cells. This is probably because it is a checkpoint gene, and has growth inhibitory properties.

We successfully produced a portion of Mec1 (encompassing the putative kinase domain) as a Glutathione-S-Transferase (GST-) fusion protein in bacteria. This polypeptide is easily purified, and will be used along with other Mec1 deletion mutants expressed in bacteria for pull-down experiments to map the sites of interaction with Rad53 and Rad9, and as a source for immunogen for antibody production. As an alternative approach, we have produced yeast expression vectors for expression of V5-tagged MEC1, and these are now under control of both *GAL1*- driven, and endogenous promoter- driven constructs. The former has been tested for the ability to complement *mec1* mutations, thus confirming the functionality of the clone. V5-expressed Mec1 is associated with protein kinase activity detectable in immune complexes. Finally, we introduced Mec1 into a two-hybrids screening vector, so that we can use two-hybrids screening to map interacting domains of Mec1 with Rad9 and Rad53.

B.Identification of Rad9/Rad53 interaction domain. *This work contributed to the publication* [3] *enclosed in the Appendix.* Using a similar logic, we endeavored to localize the domain on Rad9 that interacts with Rad53. We have already demonstrated that this interaction is a prototype for interactions of the FHA (*forkhead*-associated) protein homology domain with phospho-peptides By mapping the interaction domain with Rad9, we identified a candidate FHA target site.

The domain of Rad9 that binds to Rad53 contains a target site for Atm family protein kinases that is required for interaction with Rad53. Identification of the domain of Rad9 that interacts with Rad53 FHA2 is important for understanding this regulatory system. As a first step, we used the two-hybrids assay to localize a minimal Rad53-interacting domain (Rad9-MID). The smallest deletion mutant tested that has full binding activity contains Rad9 amino acids 542-620. Since the working hypothesis is that Rad53 FHA2 directly recognizes phospho-Rad9, and that the Rad9 kinase is Mec1, we examined the Rad9-MID for sequences characteristic of Atm family protein kinase phosphorylation sites. DNA PK preferentially phosphorylates SQ/TQ sequences, especially in an acidic environment [2, 3] The Atm sequence specificity seems similar, since two apparent in vivo targets for Atm, p53 and Abl, are both phosphorylated on sites that fit these criteria. This suggests that Mec1 would also share this target specificity. Overall, Rad9 contains 9 SQ or TQ sites, of which only one is contained within the Rad9-MID. Remarkably, this site is embedded within an acidic peptide and is nearly identical to a site on p53 phosphorylated by Atm (LSQE versus LTQE for Rad9) [4, 5] .

A site-directed mutation of the Rad9-MID replacing Rad9 T603 with A almost competely inhibited interaction in the 2 hybrids assay, even though the level of expression of the fusion protein was similar to its non-mutated counterpart . We next determined whether the Rad9-MID is a substrate for DNA PK.

GST-Rad9-MID was expressed in bacteria, purified on glutathione-agarose, cleaved from the GST tag with thrombin, and incubated with DNA PK. DNA PK phosphorylated RAD9-MID well, but failed to phosphorylate the same protein with the T603 to A substitution, despite the presence of 14 other serine and threonine residues on the same peptide. Hence these data show that T603 is required for interaction of RAD9 with Rad53, and that it is an in vitro target for a protein kinase related to Mec1.

II.Technical Objective 3. Screening based upon protein sequence homology.

We proposed to use a nested PCR strategy to identify mammalian homolog(s) of the checkpoint protein kinase Rad53. This gene, and its homologs in fission yeast and *Drosophila melanogaster* ,are characterized by the linkage of an FHA domain to a protein kinase domain. Briefly, the approach was to produce a pool of cDNAs by PCR amplification of a Jurkat cell cDNA library using a degenerate upstream primer that would recognize FHA domains, and a degenerate downstream primer that would recognize protein kinase domains. The pool was then enriched for PCR products that fall within the expected 500-1200 b.p. size range, and cloned into Bluescript SKII+. Plasmids with the appropriate-sized inserts were identified, and representative members were analyzed by nucleotide sequence and DNA database searches.

The detailed procedure and results are shown on the following page. Thus far, only a single protein kinase gene, *CHK1* has been identified. This result is intriguing, because mammalian *CHK1* had already been identified on the basis of homology to *RAD53*. However, with the clones that have been sequenced to date, it appears that priming by the degenerate FHA domain primers was non-specific. This is not a complete surprise, since the degeneracy of FHA domains and the punctate nature of conserved areas has made designing primers a major challenge. (However, the primers chosen did correctly amplify RAD53 in control experiments).

Summary of PCR Screen for FHA Protein Kinase

Step		Goal
1	primer design	design degenerate FHA and Kinase domain primers
2	reaction optimization	determine anneal temps for the all reactions
		optimize reaction to reduce background
		test against specific and nonspecific/dummy DNA
3	prepare initial template	amplify (liquid stock) lambda gt11 library
		(resting Jurkat library from S. Weissman)
		prepare clean DNA from phage
4	Reaction 1	amplify kinase domain-containing sequences
5	purify DNA	remove extra primers, primer dimers
6	Reaction 2	amplify kinase domain-containing sequences that also contain an upstream FHA domain

	DKD		
FHA	1	2	3
1	A	B	C
2	D	E	F
3	G	H	I

7	size select DNA via agarose gel	enrich for inserts of expected size
		selected ~500-1200 bp range
8	clone inserts	clone PCR products into EcoRV cut, CIP'd pBluescriptSKII+
9	size inserts of transformants	identify non-vector-only transformants by uncut size (by comparison)
	by uncut size	PCR insert (via T3/T7) to size
	by insert PCR	select 2 of each pool for sequencing based on expected and observed size

Results	major detected homology
A	vector
B	CHK1
C	APEX
D	appears identical to C2, but in opposite orientation
E	TRIP2
F	unknown
G	identical to E1
H	snurportin1
I	CHK1

III. Technical Objective 3A. Identification of authentic mammalian Rad53/Cds1

Another approach that we had proposed to use (Technical Objective 3A) was to further characterize ESTs related to *RAD53* by sequencing homologous cDNAs. In the course of this work, other research groups used this strategy to identify a mammalian homolog to the *RAD53*-related yeast protein kinase *DUN1*. This gene is denoted Chk2 or hCds1 [5-7]. Like Rad53, it is regulated by DNA damage and by replication inhibition, and its regulation requires the upstream PIKK homologs of Mec1, ATR or ATM.Chk2 is a genuine Rad53 homolog.

Year 2.

Mechanisms of Chk2 activation in response to DNA damage.

In *S. cerevisiae*, *MEC1* and *TEL1* are functionally and structurally related to the human tumor suppressor *ATM*. *MEC1* and *RAD53*, two essential genes, play a central role in DNA damage checkpoints at all cell cycle stages. Our lab showed that Rad9 is a regulator coupling DNA damage to signaling through Mec1 to activate Rad53 and Chk1 in G1 and G2 checkpoints but not in the S-phase checkpoint [4, 8]. Phosphorylation of both Rad53 and Rad9 in response to DNA damage is *MEC1*-dependent. Similarly, in mammals, activation of Chk2 requires Atm and/or Atr, the orthologs of Mec1 and Tel1. Double-stranded DNA breaks activate Atm, which in turn phosphorylates Chk2 at multiple sites, including T68. Substitution of T68 with other amino acids impairs activation of Chk2 [7, 9-11].

I.Cell-free activation of Chk2. *A manuscript describing this work has been published, and is supplied in the Appendix [12].*

Although it is clear that Atm or Atr are required for DNA damage-dependent activation of Chk2, it has not been possible to activate Chk2 or its yeast homologs by phosphorylation with either of these two enzymes or their yeast equivalents. A fortuitous observation in our laboratory has now paved the way for cell-free activation of Chk2 in a mammalian system. We found that Chk2 produced by coupled transcription/translation in a reticulocyte lysate system was phosphorylated. Phosphorylation of rabbit reticulocyte Chk2 occurs even with kinase-defective Chk2, meaning that transphosphorylation occurs in this cell-free system. Based upon caffeine and wortmannin sensitivity patterns, it appears that rabbit Atm is required for phosphorylation of Chk2 produced in vitro [12].

In contrast to Chk2 produced by translation in reticulocyte lysates, Chk2 produced by translation in a wheat germ system was not hyperphosphorylated. Importantly, this lack of phosphorylation correlated with weak in vitro kinase activity. However, incubation of wheat germ-produced Chk2 with reticulocyte lysate enabled Chk2 phosphorylation and catalytic activation. To our knowledge, this is the first mammalian cell-free system to enable catalytic activation of Chk2. Further work with this in vitro activation system will make it possible for us to determine the minimal components necessary for activation of this protein.

II.Chk2 regulation by oligomerization [12].

Earlier unpublished work in our laboratory had shown that the yeast Chk2 homolog Rad53 can interact with itself in two hybrids screens. This suggested that Chk2 oligomerization may be an important mechanism for Chk2 activation. To determine if this is the case, we measured oligomerization of Chk2 using a variety of in vivo and in vitro assays. For example, FLAG-tagged and HA-tagged Chk2 were coimmunoprecipitated when expressed in 293 cells. Importantly, the association was enhanced by DNA damage, suggesting that it is functionally relevant for the DNA damage response.

Chk2 has a N-terminal SQ/TQ cluster followed by a FHA (forkhead-associated) domain, and a C-terminal kinase domain. It differs from yeast Rad53 in that the latter (uniquely among FHA-containing protein kinases) has a second FHA domain. The Chk2 SQ/TQ cluster contains seven potential

phosphorylation sites for Atm. Threonine 68 is one major Atm phosphorylation site in response to ionizing irradiation. Earlier work in our lab showed that FHA domain-containing proteins mediate interactions with phosphoproteins through FHA domains, based on the phospho-Rad9/Rad53 interaction.

Since we had determined that DNA damage induces association of Chk2 in a complex containing other Chk2 molecules, we wondered whether interactions between Chk2 FHA domains and phospho-SQ/TQ clusters were involved. We found that for in vivo co-immunoprecipitation experiments, at least one molecule must have an FHA domain, and the other molecule must have the SQ/TQ cluster. GST-tagged Chk2 FHA domain will pull down Chk2 from DNA damaged cells, also suggesting that DNA damage-dependent phosphorylation of Chk2 promotes Chk2 oligomerization. Although these data do not preclude indirect interaction of Chk2 molecules within larger checkpoint complexes, we found that bacterially expressed forms of Chk2 will interact. When WT Chk2 is produced at high concentrations in bacteria, it undergoes partial autophosphorylation, including phosphorylation at the *trans* site T68 favored by Atm. Bacterially-produced GST-FHA domain will pull this protein down, demonstrating that homotypic interaction can occur between the Chk2 FHA domain and autophosphorylated Chk2 in the absence of other eukaryotic proteins. Finally, interactions between bacterially expressed kinase defective Chk2 (non-phosphorylated) and a His-Flag-tagged WT Chk2 (autophosphorylated) were abolished by pretreatment with lambda phosphatase. Hence, these homotypic interactions are indeed phosphorylation-dependent.

An important function of Chk2 oligomerization may be that it enables cross-phosphorylation of Ch2k molecules, leading, in turn, to full activation of Chk2. We found, using in vitro kinase assays containing bacterially produced kinase-defective GST-Chk2 and WT His-FLAG-Chk2, that transphosphorylation of the kinase-defective molecule could occur, including phosphorylation at T68.

In summary, we have shown that DNA damage can induce oligomerization of Chk2 in vivo, and that such complexes require both Chk2 FHA domains and phosphorylated Chk2 SQ/TQ clusters. The interactions are phosphorylation-dependent, and can be enabled by Chk2 autophosphorylation at T68, a major trans-regulatory target for Atm. We conclude that Atm-dependent phosphorylation of Chk2 may be a priming event that permits further autophosphorylation and dimerization of Chk2. Once activated, Chk2 may be able to activate other quiescent Chk2 molecules by transphosphorylation at T68, *even in the absence of functional Atm*. These phospho-Chk2 molecules may also assemble into oligomers, in large signaling complexes [12].

III.Proteins targeted for phosphorylation by Chk2.

Known effector substrates for Chk2 include Cdc25A, Cdc25C, and p53 [7, 13, 14]. The latter two proteins are also targeted by the structurally distant kinase Chk1, which operates in parallel to Chk2 in many organisms. A major goal is to identify new substrates for Chk2, some of which may turn out to be Chk2-specific. In order to begin this work, we produced a number of reagents:

- -GST-Chk2 and GST-Chk2 D368A (kinase-inactive) proteins. They have been examined by *in vitro* kinase assays for their activities. GST-Chk2 can autophosphorylate by incorporation of 32P γ-ATP, while GST-Chk2D368A cannot.
- HA-Chk2 and HA-Chk2 D368A (kinase-inactive) plasmids which have been used for transient transfections and for stable transfection of HT-1080 cells (with mutated p53). Chk2 proteins immunoprecipitated from those cells were used for *in vitro* kinase assays for autophosphorylation to examine the kinase-inactive mutant (successfully) and for trans-phosphorylation of p53 and RPA - unsuccessfully so far.
- Preparation of α-GST-Chk2 polyclonal rabbit antibodies. Those antibodies both immunoprecipitate and immunoblot Chk2.
- Phosphospecific antibody for P-T68 in Chk2. 16 AA oligopeptide that resembles a region around phosphorylated T68 in Chk2 has been cross-linked to KLH protein and used for immunization of two rabbits. We have verified that the antiserum produced recognizes Chk2 protein only when it is phosphorylated on T68 (active form). This phosphorylation is probably mediated in vivo by Atm, and Atr.

Year 3.

I.Chk2 and RPA

One of the groups of potential Chk2 substrates is a subset of proteins that undergo ATM-dependent phosphorylation after exposure of cells to IR. One of those proteins is the middle subunit of replication protein A (Rpa2). Human RPA2 is also heavily phosphorylated in response to DNA damage. Unpublished work from our laboratory had suggested the possibility that the DNA replication factor RPA2 in yeast is a substrate for Rad53. Hence, we determined if RPA and Chk2 can interact. We found that a small population[15] of RPA2 co-immunoprecipitates with HA-Chk2 by IP with α-Chk2 and α-HA antibodies.

We have performed a kinase assay with GST-Chk2 and Rpa, generously provided by Bruce Stillman's laboratory. We found that GST-Chk2 phosphorylates Rpa1 and Rpa2, but GST-Chk2-KD does not. We investigated by co-immunoprecipitation if Rpa and Chk2 can interact. We have found that a small subpopulation of RPA2 co-immunoprecipitates with HA-Chk2 by IP with anti-Chk2 and anti-HA antibodies.

To determine if Rpa2 phosphorylation after DNA damage is Chk2-dependent we performed several experiments. Treatment of HT-1080 cells with 1 mM camptothecin (CPT), a topoisomerase I inhibitor, for 1 hour causes Rpa2 hyperphosphorylation, which can be prevented by 15min pre-treatment of cells with 5mM a protein kinase inhibitor UCN-01 (7-hydroxystaurosporine). The concentration of UCN-01that prevents Rpa2 phosphorylation is quite different from the one that inhibits Chk1 (IC50=15nM). Also, we have found that treatment of HT-1080 cells with 1mM CPT causes gel mobility shift of Chk2, which can be diminishes by pre-treatment of cells with 1-5mM UCN-01.

Experiments with pre-treatment of HT-1080 cells, stably transfected with pcDNA3-HA-Chk2, with different concentrations of UCN-01 showed that dose/ response curves of Rpa2 and Chk2 phosphorylation in response to treatment with 1mM CPT are similar. To determine if the Rpa2 and Chk2 phosphorylation are ATM-dependent, we transiently transfected AT cells with pcDNA3-HA-Chk2 and treated those cells

with CPT. Chk2 and RPA2 proteins were shifted, so we concluded that those responses are *ATM*-independent. Other possible candidate kinases are Atr and DNA-PK, which we can distinguish by treatment of AT cells with different concentration of protein kinase inhibitors caffeine and wortmannin. We pre-treated AT cells, transiently transfected with HA-Chk2 plasmid, with 10m wortmannin or 3mM caffeine for 20 min, and treated later with 1mM CPT. Chk2 and Rpa2 phosphorylation was caffeine-sensitive and wortmannin-insensitive, so most probably this pathway goes through Atr rather than DNA-PK.

To determine if Rpa2 phosphorylation is Chk2-dependent we treated HT-1080 cells, stably transfected with HA-Chk2 and HA-Chk2D368A (KD-dominant negative mutant), with CPT and then measured Rpa2 hyperphosphorylation (mobility shift in the gel) by immunoblotting. We did not detect a decrease of Rpa2 hyperphosphorylation in cells stably transfected with dominant-negative Chk2 mutant.

In summary, we have found that Chk2 can phosphorylate the large and middle subunits of Rpa *in vitro*. Also, we have observed a physical interaction between Chk2 and Rpa2 by co-immunoprecipitation. The data associating phosphorylation of Chk2 and Rpa2 after treatment with CPT are correlative only and it will be necessary to establish a better system to verify the Chk2-dependence of Rpa2 phosphorylation.

II. Chk2 and Nibrin.

We have observed that Chk2 functionally interacts with the Nibrin-Rad50-Mre11 complex:

1) Chk2 coimmunoprecipitates with nibrin in 293 cells and HT1080 cells. We generated a series of deletion mutants of Chk2 and nibrin. These mutants will be used to identify the domains responsible for this interaction. We also generated GST fusion proteins with overlapping fragments of nibrin. These GST proteins will be used in kinase assays to determine if Chk2 is upstream of nibrin.
2) Chk2 coimmunoprecipitates with Rad50, but not Mre11.
3) Restoration of expression of nibrin in the nibrin-deficient cell line (GM07166) results in Chk2 hyperphosphorylation in response to ionizing irrdiation. Basal levels of phosphorylation of Chk2 are seen in GM07166, but not in GM07166 transfected with NBS1 cDNA and irradiated. We have generated point mutants knocking out all the potential phosphorylation sites within the SQ/TQ cluster of Chk2, and add-back mutants retaining one potential phosphorylation site within the SQ/TQ cluster of Chk2. These mutants will help identify the phosphorylation sites in Chk2, which are nibrin-dependent, in response to DNA damage.

III. Chk2/Nibrin complexes.

We also observed that Chk2 forms a large protein complex:

1) Chk2 forms an oligomer, since HA tagged Chk2 coimmunoprecipitates FLAG-tagged Chk2 in 293 cells. This is consistent with our earlier work on Rad53, since a two-hybrid screen with Rad53 as bait pulled out Rad53
2) Our preliminary gel filtration experiments indicate that the Chk2-containing complex is in the range of 500 – 700 kDa. Chk2 co-fractionates with nibrin, Rad50, and Brca1. We are in the process of purifying this Chk2-containing complex.

Our observations indicate three possible models of the functional relationships between Chk2 and the nibrin-Rad50-Mre11 complex:

1) the nibrin-Rad50-Mre11 complex is upstream of Chk2 and regulates Chk2 activation in checkpoint controls
2) Chk2 is upstream of the nibrin-Rad50-Mre11 complex and regulates DNA repair
3) Chk2 may be essential for both checkpoint controls and DNA repair.

Two groups have now demonstrated that Nbs1 is required for fully Chk2 activation in response to DNA damage [15]. Nevertheless, our observation that Chk2 interacts with and phosphorylates Nbs1 *in vitro* indicates that Chk2 may regulate Nbs1 function as well.

Year 4 (no-cost extension).

I.Chk2 and Plk.

In attempt to identify new Chk2 interacting proteins we exploited the homology between yeast and mammalian DNA damage pathways. From genetic studies in yeast DNA damage checkpoint pathway it is known that RAD53 genetically interacts with CDC5, a gene for Polo-like kinase [16]. After DNA damage Cdc5 is phosphorylated in Rad53-dependent manner. On the other hand CDC5 turns-off Rad53 in a process of adaptation to unrepairable DNA damage [17]. The human homolog of CDC5 Polo-like kinase 1 is a multifunctional mitotic protein kinase involved in many mitotic events, including centrosome maturation and formation of the mitotic spindle. Also, Plk1 is a target of DNA damage checkpoint and it is inhibited after DNA damage in ATM-dependent manner [18-21]..

We examined by immunoprecipitation whether Chk2 and Plk1 interact. Extracts from 293-T cells, transiently transfected with plasmids for HA-tagged Chk2 and Flag-tagged Plk1, were IP with HA and Flag antibodies. Immunoblot analysis of the immunoprecipitates showed that HA-Chk2 and Flag-Plk1 physically interact. To confirm this interaction we immunoprecipitatied Chk2 from whole cell extract from 293 cells and detected Plk1 in Chk2 immunoprecipitates by immunoblotting.

To further characterize the interaction between Chk2 and Plk1, we created plasmids expressing deletion mutants of HA-Chk2 and Flag-Plk1. Mutant HA-Chk2 and Flag-Plk1 proteins were expressed in 293-T cells and co-IP with HA and Flag antibodies were performed. The data from immunoblot analysis of immunoprecipitates showed some modulation of Chk2/Plk1 interaction, although we could not identify a single domain in the proteins critically important for their physical interaction. This fact could be explained by a multiplicity of contacts within the Chk2/Plk1 complex.

To investigate further the interaction between Chk2 and Plk1, we tested if they co-localize in the cell. Plk1 is expressed in S, G2 and M phases of cell cycle and its activity peaks in M phase. Plk1 is associated with the kinetochores of condensed chromosomes, the centrosome in prophase and metaphase, and the midbody, later in mitosis. From the work of others it was known that Chk2 is localized mainly in the nucleus. Phosphorylation of Chk2 at several SQ/TQ sites in its N-terminal part is required for Chk2 activation after DNA damage. ATM prefers one of this sites-Thr68 which phosphorylation is required for Chk2 DNA damage-dependent activation [22, 23]. To track the location of active Chk2 we raised and affinity purified a phospho-specific antibody, raised against phosphopeptide resembling the region around phosphoThr68, that recognized Chk2 only when it is phosphorylated at Thr68. The specificity of the antibody was examined by immunoblot analysis. PT68-Chk2 recognized endogenous Chk2 and transiently expressed HA-Chk2 from 293-T cells irradiated with IR but not from mock-irradiated cells. The antibody did not recognize transiently expressed mutant HA-Chk2T68A neither from irradiated nor from non-irradiated cells.

At the sites of DNA damage, Chk2 is phosphorylated at Thr68 and formed immunoreactive foci. To test if our antibody can detect these foci we immunostained 293-T, WI-38, HT-1080 and GM5849C cells irradiated with 0 Gy or 4 Gy gamma radiation. We observed foci formation following exposure to ionizing radiation in 293-T, WI-38 and HT-1080 cells but not in GM5849C cells, an AT cell line .

(7) KEY RESEARCH ACCOMPLISHMENTS.

Major accomplishments include production of key reagents for work on Chk2 and other human checkpoint proteins, surveys of possible Chk2 interactors and substrates including Nibrin, RPA, PLK, and KIAA1070, partial elucidation of phospho-Rad9/Rad53 interactions, and proposal of a new model for activation of Chk2 by DNA damage. Technical achievements include:

- Expression of functional Mec1, and identification of associated protein kinase activity.
- Identification of a domain on Rad9 that is required for interaction with Rad53 [3]
- Identification of a putative phosphorylation target site for Atm family members that is likely to be the target for the Rad53 FHA domain [3]
- Completion of first round screens for FHA-containing protein kinase genes
- Development of antibodies and epitope-tagged clones for analysis of Chk2, and Brct domain containing proteins Brca1, Nibrin, 53BP1, and KIAA0170. These antibodies include a phospho-specific antibody that exclusively recognizes active Chk2.
- Investigation of requirements for Atm, Nibrin, and Brca1 in regulation of Chk2.
- Evaluation of Chk2 association with Brca1, Nibrin, 53BP1, KIAA0170, Rad50.
- Production of deletion and other mutants of Nibrin for investigating Nibrin interactions with Chk2.
- Production of cell lines expressing wildtype Chk2 and a number of phosphorylation site and other mutants.
- Investigation of RPA as a new Chk2 substrate.
- Preparation of molecular clones to be used for recombinational inactivation of human *CHK2* in somatic cells.
  - Our work lends further credence, but not proof, to the hypothesis that RPA is a substrate for Chk2 in vivo.
  - Our work has identified a novel ability of Chk2 to phosphorylate itself at T68, and to form dimers as a result of this autophosphorylation. On this basis, we have proposed a novel model for Chk2 activation [12]
  - We have developed the first mammalian cell-free system to enable catalytic activation of Chk2. This system will enable us to identify factors required for Chk2 activation [15].

Connection with Approved Technical Objectives.

Technical Objective 1. Protein-interaction screens for mammalian Rad53 and Rad9 homologs.
partly completed- obviated with cloning of Chk2 in year 1
Technical Objective 2. Cloning of mammalian DNA checkpoint genes by complementation of defects in yeast.
obviated with cloning of Chk2
Technical Objective 3. Screening based upon protein sequence homology.
partly completed, obviated with cloning of Chk2
Technical Objective 4. Characterization of genes in mammalian cells.
substantial progress as detailed above

(8) REPORTABLE OUTCOMES.

- Work funded by this grant was presented in poster form at the USAMRMC ERA of HOPE Meeting held in Atlanta, May, 2000. Interaction of Proteins that Protect Cells from DNA Damage. Marc F. Schwartz., Dr. Zhaoxia Sun, James Hsiao, and Dr. David F. Stern

- Work funded by this grant will be presented in posters from Lyuben Tsvetkov and Xavier Xu (with David F. Stern) at the forthcoming ERA of HOPE Meeting to be held in Orlando in September, 2002.

- Work supported by this grant has contributed to two major publications, with more expected as work in progress is completed:

Schwartz, M. F., J. K. Duong, et al. (2002). "Rad9 phosphorylation sites couple Rad53 to the Saccharomyces cerevisiae DNA damage checkpoint." Mol Cell **9**(5): 1055-65.

Xu, X., L. Tsvetkov, et al. (2002). "Chk2 activation and phosphorylation-dependent oligomerization." Mol.Cell.Biol. **22**: 4419-4432.

(9) CONCLUSIONS

Alterations in DNA damage response pathways, including checkpoint pathways, are known to occur frequently in human carcinogenesis. Such changes in breast cancer include mutations in p53, Brca1, and Brca2. Genetic analysis of yeasts has identified DNA checkpoint pathways that are necessary to maintain the genome in the face of DNA damage. The identification of the human ATM gene, which is homologous to a major element in yeast checkpoint pathways, led to the hypothesis that other member of these checkpoint pathways would also be conserved in mammals. The purpose of the research that we conducted has been to, first, identify such human homologs, and second, determine how they function in mammalian checkpoint pathways. Our work in the first year of the research laid the groundwork for identification of proteins that are functionally related to the yeast checkpoint protein Rad9. Since Mec1/Rad9/Rad53 interactions are a prototype for an important machine in the checkpoint control apparatus, identification of a Rad53 interaction domain of Rad9 (which later turned out to be one of several) represented a major step forward in understanding of this machinery. Moreover, the Rad9 site represents the first identified phosphopeptide that interacts with a FHA domain. This phosphopeptide/FHA interaction is itself the paradigm for function of all FHA domains, and represents a major advance in understanding signal transduction by these modules

As we had hypothesized, the major components of DNA checkpoint pathways have been shown to be conserved between budding yeast and humans. At the time of submission of the original proposal, the only human ortholog of a yeast checkpoint gene was Atm. Since then, this list has been extended to the genes listed in Table 1, which includes a homolog of yeast Rad53, which we originally sought. With the identification of the yeast Rad53 homolog, Chk2/Cds1, we have investigated how Chk2 is regulated, and how it regulates targets. This work is directly relevant to breast cancer, since it is now clear that Chk2 is an intermediary linking DNA checkpoint pathways from Atm to p53; since Chk2 phosphorylates and modulates Brca1 function and since Chk2 mutations are found in variant p53+ forms of Li-Fraumeni syndrome, which predisposes to breast cancer and other cancers [24]. There is also an elevated breast cancer risk associated with certain forms of Chk2, and there may be somatic mutations in other cancers [25, 26] [2].

Work on these genes and pathways is not simply an academic exercise. These DNA checkpoint proteins have important roles in breast cancer. Not only will progress on these problems enhance our understanding of carcinogenic processes, they may be important for optimization of breast cancer therapies. The response of tumor cells to genotoxic chemotherapeutic agents is greatly affected by the impact they have on the DNA checkpoint pathways including Chk2. It can be anticipated that fruits of this work will include better therapeutic modulators, and the ability to maximize utility of existing therapies.

(10) REFERENCES.

1. Zhou, B.B. and S.J. Elledge, *The DNA damage response: putting checkpoints in perspective.* Nature, 2000. **408**(6811): p. 433-9.
2. Bartek, J., J. Falck, and J. Lukas, *CHK2 kinase--a busy messenger.* Nat Rev Mol Cell Biol, 2001. **2**(12): p. 877-86.
3. Schwartz, M.F., et al., *Rad9 phosphorylation sites couple Rad53 to the Saccharomyces cerevisiae DNA damage checkpoint.* Molecular Cell, 2002. **9**: p. 1055-1065.
4. Sun, Z., et al., *Rad53 FHA domain associated with phosphorylated Rad9 in the DNA damage checkpoint.* Science, 1998. **281**: p. 272-274.
5. Matsuoka, S., M. Huang, and S.J. Elledge, *Linkage of ATM to cell cycle regulation by the Chk2 protein kinase.* Science, 1998. **282**(5395): p. 1893-7.
6. Brown, A., et al., *A human Cds1-related kinase that functions downstream of ATM protein in the cellular response to DNA damage.* Proc Natl Acad Sci U S A, 1999. **96**: p. 3745-3750.
7. Blasina, A., et al., *A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase.* Curr Biol, 1999. **9**: p. 1-10.
8. Sun, Z., et al., *Spk1p is regulated by MEC1-dependent protein phosphorylation in DNA-replication and damage checkpoint pathways.* Genes Dev., 1996. **10**: p. 395-406.
9. Matsuoka, S., M. Huang, and S. Elledge, *Linkage of ATM to cell cycle regulation by the Chk2 protein kinase.* Science, 1998. **282**: p. 1893-1897.
10. Chaturvedi, P., et al., *Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway.* Oncogene, 1999. **18**(28): p. 4047-54.
11. Lee, C.H. and J.H. Chung, *The hCds1 (Chk2)-FHA domain is essential for a chain of phosphorylation events on hCds1 that is induced by ionizing radiation.* J Biol Chem, 2001. **276**(32): p. 30537-41.
12. Xu, X., L. Tsvetkov, and D.F. Stern, *Chk2 activation and phosphorylation-dependent oligomerization.* Mol.Cell.Biol., 2002. **22**: p. 4419-4432.
13. Falck, J., et al., *The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis.* Nature, 2001. **410**(6830): p. 842-7.
14. Chehab, N.H., et al., *Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53.* Genes Dev, 2000. **14**(3): p. 278-88.
15. Buscemi, G., et al., *Chk2 activation dependence on Nbs1 after DNA damage.* Mol Cell Biol, 2001. **21**(15): p. 5214-22.
16. Sanchez, Y., et al., *Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms.* Science, 1999. **286**(5442): p. 1166-71.
17. Toczyski, D.P., D.J. Galgoczy, and L.H. Hartwell, *CDC5 and CKII control adaptation to the yeast DNA damage checkpoint.* Cell, 1997. **90**(6): p. 1097-106.
18. Nigg, E.A., *Polo-like kinases: positive regulators of cell division from start to finish.* Curr Opin Cell Biol, 1998. **10**(6): p. 776-83.
19. Smits, V.A., et al., *Polo-like kinase-1 is a target of the DNA damage checkpoint.* Nat Cell Biol, 2000. **2**(9): p. 672-6.
20. van Vugt, M.A., et al., *Inhibition of Polo-like kinase-1 by DNA damage occurs in an ATM- or ATR-dependent fashion.* J Biol Chem, 2001. **276**(45): p. 41656-60.
21. Toyoshima-Morimoto, F., E. Taniguchi, and E. Nishida, *Plk1 promotes nuclear translocation of human Cdc25C during prophase.* EMBO Rep, 2002. **3**(4): p. 341-8.

22. Melchionna, R., et al., *Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1.* Nat Cell Biol, 2000. **2**(10): p. 762-5.
23. Matsuoka, S., et al., *Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro.* Proc Natl Acad Sci U S A, 2000. **97**(19): p. 10389-94.
24. Bell, D., et al., *Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome.* Science, 1999. **286**: p. 2528-25431 (see later correction in Science).
25. Meijers-Heijboer, H., et al., *Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations.* Nat Genet, 2002. **31**(1): p. 55-9.
26. Miller, C.W., et al., *Mutations of the CHK2 gene are found in some osteosarcomas, but are rare in breast, lung, and ovarian tumors.* Genes Chromosomes Cancer, 2002. **33**(1): p. 17-21.

(11) Bibliography.

Abstracts.

USAMRMC ERA of HOPE Meeting May, 2000. Interaction of Proteins that Protect Cells from DNA Damage. Marc F. Schwartz., Dr. Zhaoxia Sun, James Hsiao, and Dr. David F. Stern

Publications.

Schwartz, M. F., J. K. Duong, et al. (2002). "Rad9 phosphorylation sites couple Rad53 to the Saccharomyces cerevisiae DNA damage checkpoint." Mol Cell **9**(5): 1055-65.

Xu, X., L. Tsvetkov, et al. (2002). "Chk2 activation and phosphorylation-dependent oligomerization." Mol.Cell.Biol. **22**: 4419-4432.

(12) List of Personnel

The following individuals have been partly supported by funds from this proposal:

David F. Stern, PI
Xingzhi Xu, Postdoctoral Fellow
Mary Davis, Glassware
Rajani Ramabhadran, Research Associate

The following individuals have performed experimental work supported by this grant, but received salary support from other sources:

Marc Schwartz, Graduate Student
Lyuben Tsvetkov, Postdoctoral Fellow

	Saccharomyces cerevisiae	*Schizosaccharomyces pombe*	*Homo sapiens*	
	Mec1 Tel1	rad3#	Atr Atm	PI kinase family; protein kinase
	Ddc2?	rad26 (*P)#		
	DAMAGE PATHWAY			
	Rad9 (*P)	Crb2/Rhp9(*P)	Brca1? (*P) p53BP1? Nibrin? KIAA0170?	**BRCT** domains
	Rad24	Rad17#	Rad17 (RFC4?)	
	Mec3/Pip3			"
		Hus1# *P	**Hus1**	PCNA-like complex
	Rad17	**Rad1#**	**rad1**	"
	Ddc1 (*P)	**Rad9 #**	**Rad9**	"
				"
	DPB11	cut5		**BRCT** domains replication upstream Rad53
	Sld2/DRC1			replication upstream Rad53
	POL2		Pol	replication upstream Rad53
	RFC5		RFC	replication
	PRI4		Pol	replication Polα-Primase downstream Rad53
	Rad53 *P	cds1 *P	cds1/Chk2*P	protein kinase **FHA** domain(s)
	Chk1	Chk1*P	Chk1*P	protein kinase

Table 1. Conservation of yeast and mammalian DNA checkpoint genes. *P indicates that damage induces protein phosphorylation. Reviewed in [1, 2].

Molecular Cell, Vol. 9, 1055–1065, May, 2002, Copyright ©2002 by Cell Press

Rad9 Phosphorylation Sites Couple Rad53 to the *Saccharomyces cerevisiae* DNA Damage Checkpoint

Marc F. Schwartz,[1,2] Jimmy K. Duong,[2] Zhaoxia Sun,[2,3,5] Jon S. Morrow,[2] Deepti Pradhan,[2] and David F. Stern[2,4]
Departments of Cell Biology[1] and Pathology[2]
Yale University School of Medicine
[3]Department of Molecular, Cellular, and Developmental Biology
Yale University
New Haven, Connecticut 06510

Summary

Rad9 is required for the *MEC1/TEL1*-dependent activation of *Saccharomyces cerevisiae* DNA damage checkpoint pathways mediated by Rad53 and Chk1. DNA damage induces Rad9 phosphorylation, and Rad53 specifically associates with phosphorylated Rad9. We report here that multiple Mec1/Tel1 consensus [S/T]Q sites within Rad9 are phosphorylated in response to DNA damage. These Rad9 phosphorylation sites are selectively required for activation of the Rad53 branch of the checkpoint pathway. Consistent with the in vivo function in recruiting Rad53, Rad9 phosphopeptides are bound by Rad53 forkhead-associated (FHA) domains in vitro. These data suggest that functionally independent domains within Rad9 regulate Rad53 and Chk1, and support the model that FHA domain-mediated recognition of Rad9 phosphopeptides couples Rad53 to the DNA damage checkpoint pathway.

Introduction

DNA checkpoints attenuate the mutagenicity, genomic instability, and cell lethality resulting from DNA damage. In response to DNA damage, these pathways delay cell cycle progression, increase the transcription of DNA checkpoint, replication, and repair genes, and can activate apoptosis. In addition, DNA checkpoint pathways may directly regulate the DNA repair process. Several checkpoint pathway components are recognized as tumor suppressors, and dysfunction of checkpoint signaling pathways is frequently associated with cancers (reviewed in Zhou and Elledge, 2000).

DNA checkpoint pathways amplify and transmit the checkpoint signal to the effector pathways through an evolutionarily conserved kinase cascade. The first tier of this cascade involves members of a family of phosphatidylinositol-(3′) kinase-like protein kinases (PIKKs) that includes mammalian *ATM*, *ATR*, and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs). These PIKKs regulate the activation of two unrelated effector checkpoint kinases (Chks), represented by mammalian Chk1 and Chk2 (reviewed in Rhind and Russell, 2000; Durocher and Jackson, 2001; Abraham, 2001).

In *Saccharomyces cerevisiae*, the PIKK to Chk kinase cascade is an obligatory component of two DNA checkpoint signaling pathways. The DNA damage checkpoint pathway is sensitive to various forms of DNA damage throughout the cell cycle. The DNA replication checkpoint pathway functions in S phase and is activated in response to both inhibition of DNA synthesis and DNA damage (reviewed in Elledge, 1996). Although both of these pathways involve the PIKKs Mec1 or Tel1, the DNA replication checkpoint pathway activates the Chk2 homolog Rad53 but not Chk1. By contrast, the DNA damage checkpoint pathway activates both Rad53 and Chk1 (Sun et al., 1996; Sanchez et al., 1996, 1999). This differential regulation is partially mirrored in fission yeast, where the PIKK Rad3 is required for all DNA checkpoint pathways, and DNA damage activates Chk1 while replication inhibition activates the Chk2 homolog Cds1 (Walworth et al., 1993; al-Khodairy et al., 1994; Bentley et al., 1996; Lindsay et al., 1998).

S. cerevisiae RAD9 is the prototype DNA damage checkpoint gene (Weinert and Hartwell, 1988) and is required for the DNA damage checkpoint pathway throughout the cell cycle (Weinert and Hartwell, 1988; Siede et al., 1993; Paulovich et al., 1997). Rad9 shares localized homology with the mammalian tumor suppressor *BRCA1* (Koonin et al., 1996; Bork et al., 1997). Loss of *RAD9* impairs checkpoint-induced cell cycle arrest and increases genomic instability (Weinert and Hartwell, 1988, 1990). *RAD9* is required for the activation of Rad53 and Chk1 by the DNA damage checkpoint pathway (Navas et al., 1996; Sanchez et al., 1999). Moreover, DNA damage but not replication block induces Rad9 phosphorylation in a Mec1/Tel1-dependent manner, and Rad53 specifically interacts with phosphorylated Rad9 in vivo (Sun et al., 1998; Emili, 1998; Vialard et al., 1998). These data suggested that Rad9 acts as a pathway-specific adaptor that recruits Rad53 and Chk1 to DNA damage-dependent activation complexes (Sun et al., 1998; Sanchez et al., 1999). This model is supported by the recent observation that immunoprecipitated phosphorylated Rad9 facilitates the activation of exogenous Rad53 in vitro (Gilbert et al., 2001).

Rad53 and other Chk2 orthologs are coupled to DNA checkpoint pathways through their FHA domains (Sun et al., 1998; Bell et al., 1999; Chehab et al., 2000; Lee and Chung, 2001). FHA domains are conserved modular domains that bind specific phosphopeptides (Hofmann and Bucher, 1995; Sun et al., 1998; Durocher et al., 1999; Li et al., 1999; Liao et al., 1999). FHA domains were first implicated as phosphorylation-specific protein-protein binding domains in a study of the binding of *Arabidopsis thaliana* kinase-associated protein phosphatase (KAPP) to a receptor-like kinase (RLK) (Stone et al., 1994). Rad53 contains two FHA domains, which are hypothesized to couple Rad53 to the DNA checkpoint pathways (Sun et al., 1998). Both Rad53 FHA domains can bind phosphorylated Rad9 in vitro (Sun et al., 1998; Durocher et al., 1999), and mutation of conserved amino acids in the

[4]Correspondence: df.stern@yale.edu
[5]Present address: Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

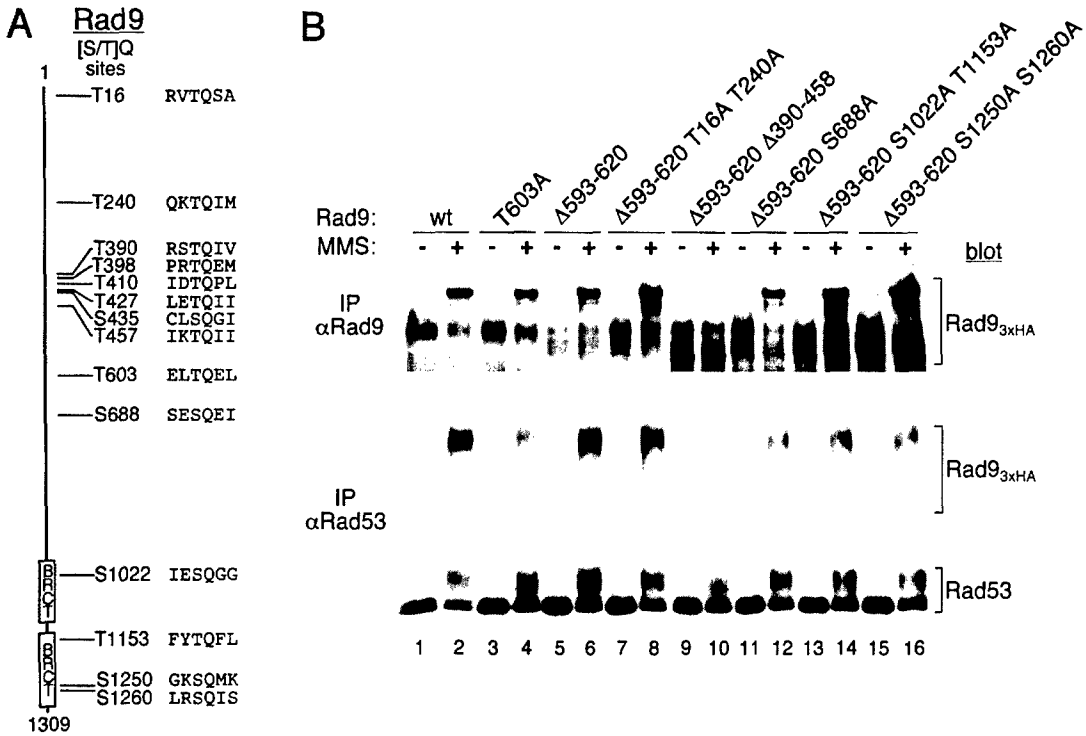


Figure 1. Mutagenesis of Rad9 PIKK Phosphorylation Sites

(A) Schematic diagram of Rad9: the tandem BRCT domains are boxed, hash marks indicate the locations of the fourteen [S/T]Q consensus PIKK phosphorylation sites, and the sequence describes ± two residues surrounding each [S/T]Q site.

(B) Anti-HA (top and middle) and anti-Rad53 (bottom) immunoblot analyses of anti-Rad9 (top) and anti-Rad53 (middle and bottom) immunoprecipitates from the indicated *RAD9* or *rad9* DLY408-derived strains. Asynchronous cultures were either mock treated (−) or treated with 0.1% MMS (+) for 1 hr.

second Rad53 FHA domain (FHA2) disrupts the DNA damage checkpoint pathway activation of Rad53 in vivo (Sun et al., 1998).

Phosphopeptide binding preferences of FHA domains have been identified in vitro using peptide libraries (Durocher et al., 2000; Liao et al., 2000; Wang et al., 2000; Byeon et al., 2001). Despite the requirement for FHA domains in multiple signaling pathways, including the Rad9/Rad53 interaction, the specific phosphopeptides recognized by FHA domains in vivo are not known. We report here the identification of multiple PIKK sites within Rad9 that are phosphorylated in response to DNA damage and are required for the *RAD9*-dependent regulation of Rad53.

Results

Identification of the Rad53 Binding Sites within Rad9

We had previously identified an interaction between Rad53 FHA2 and Rad9 in a two-hybrid assay (Sun et al., 1998). Deletion analysis revealed that Rad9 residues 542–620 are minimally required for this interaction (data not shown). This domain contains a single consensus PIKK phosphorylation site, [S/T]Q (Anderson and Lees-Miller, 1992; Kim et al., 1999), at Rad9 T603 (Figure 1A). Mec1 phosphorylates this site in immune-complex kinase assays (data not shown). Alanine substitution or deletion of Rad9 T603 did not eliminate the DNA damage-induced Rad9 phosphorylation, coimmunoprecipitation of Rad9 with Rad53, or the *RAD9*-dependent function of the G_2/M DNA damage checkpoint arrest (Figure 1B, lanes 1–6, and data not shown). Hence, Rad9 T603 is not essential for Rad9 phosphorylation and recruitment of Rad53.

To determine whether other Rad9 [S/T]Q sites are required for DNA damage-dependent Rad9 phosphorylation and its interaction with Rad53, we generated a series of strains lacking Rad9 T603 in combination with either alanine substitutions or deletion of these sites (Figure 1B, lanes 7–16). Of these mutations, only the additional deletion of the [S/T]Q cluster domain (SCD), amino acids 390–458, impaired the DNA damage-induced phosphorylation of Rad9 (Figure 1B, lanes 9–10). Furthermore, Rad9 and Rad53 did not coimmunoprecipitate in this strain, and Rad53 phosphorylation was partially impaired (Figure 1B, lanes 9–10). As this experiment used asynchronous cultures, the residual Rad53 phosphorylation observed was probably mediated by the *RAD9*-independent DNA replication checkpoint pathway. The single deletion of Rad9 amino acids 390–458 also abrogated the DNA damage-induced phosphorylation of Rad9 (Figure 2A).

Since deletions within Rad9 may nonspecifically disrupt Rad9 function, we examined the effects of alanine substitution at the [S/T]Q sites within the SCD and at T603. The *rad9*^{6xA} allele has alanine substitutions at each [S/T]Q within the SCD (T390, T398, T410, T427, S435, and T457), and the *rad9*^{7xA} allele is the *rad9*^{6xA} allele with

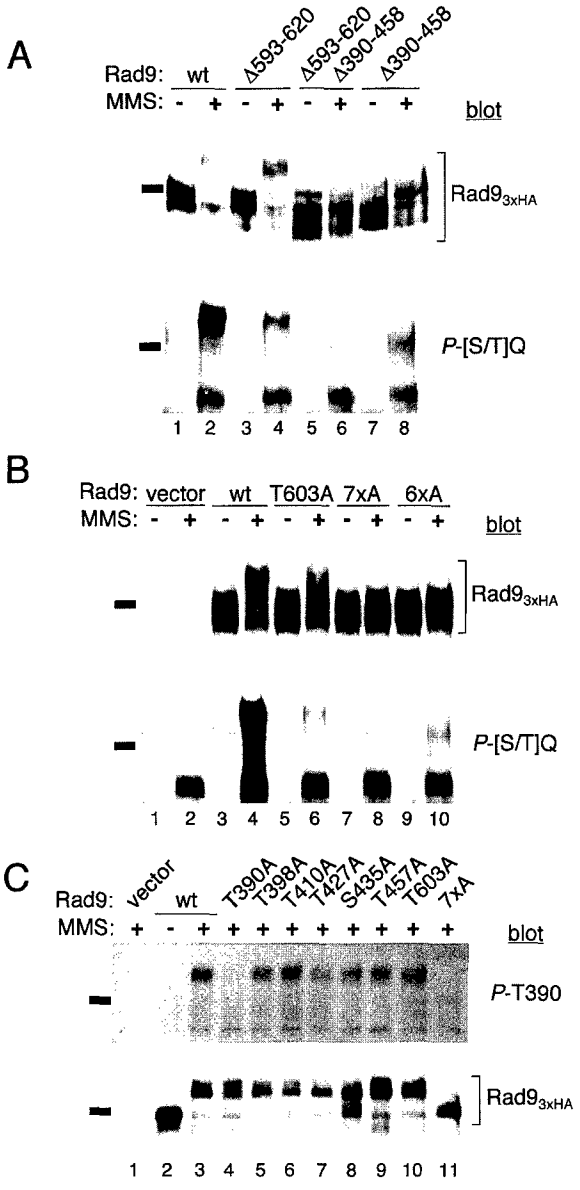


Figure 2. DNA Damage Induces Rad9 Phosphorylation at Multiple [S/T]Q Sites

In (A) and (B), duplicate blots of TCA lysates from asynchronous cultures of (A) DLY408-derived and (B) DLY418-derived strains were immunoblotted with anti-HA (top panels) and anti-phospho-[S/T]Q (bottom). In (C), a blot of TCA lysates from *cdc15-2* synchronized DLY418-derived strains was immunoblotted with anti-phospho-T390 (top panel) and anti-HA (bottom panel). The bar to the left of the blots indicates the position of the 207 kDa marker. Cells were either mock treated (−) or treated with 0.1% MMS (+) for 1 hr.

the additional alanine substitution of T603. Compared to wild-type Rad9, both Rad9^{7xA} and Rad9^{6xA} displayed severely reduced DNA damage-dependent electrophoretic mobility shift (Figure 2B), indicating that these mutations eliminate the majority of Rad9 phosphorylation sites or otherwise impair the damage-dependent Rad9 phosphorylation.

Multiple forms of DNA damage induce the *MEC1/TEL1*-dependent phosphorylation of Rad9 (Emili, 1998). Since it is not known whether these forms of phosphorylated Rad9 involve different combinations of phosphorylation sites, we examined the effect of Rad9 SCD mutations on the mobility shift of Rad9 induced by different types of DNA damage. Rad9^{6xA} was similarly impaired for phosphorylation and Rad53 coimmunoprecipitation induced by DNA damage from MMS, 4NQO, and UV and ionizing radiation (data not shown). Hence, the Rad9 SCD is a major locus for both Rad9 phosphorylation and interaction with Rad53 induced by DNA damage.

Detection of Phosphorylated Rad9 [S/T]Q Sites, Including T603 and SCD Site T390

DNA damage-dependent Rad9 phosphorylation requires the PIKKs Mec1 or Tel1 (Sun et al., 1998; Emili, 1998; Vialard et al., 1998). Rad9 contains a total of fourteen [S/T]Q consensus PIKK phosphorylation sites, including T603 (Figure 1A). Immunoblot analysis with an antibody that recognizes phosphorylated [S/T]Q sites revealed a strong immunoreactive band that is induced by DNA damage, is eliminated by deletion of *RAD9*, and comigrates with phosphorylated Rad9 (Figure 2B, lanes 1–4). According to the manufacturer, this antibody preferentially recognizes phospho-[S/T]Q when preceded by a leucine or other hydrophobic residue. Thus, given its context, Rad9 T603 would be an ideal binding substrate if phosphorylated. Indeed, although Rad9$^{\Delta 593-620}$ and Rad9^{T603A} undergo apparently normal phosphorylation-dependent electrophoretic mobility shifts, their phospho-[S/T]Q immunoreactivity was significantly reduced (Figure 2A, lanes 3–4; Figure 2B, lanes 5–6). This residual phospho-[S/T]Q immunoreactivity was eliminated with the additional loss of the Rad9 SCD, but was partially recovered in the Rad9 mutant only for the SCD (Figure 2A, lanes 5–8; Figure 2B, lanes 7–10). Together, these data imply that DNA damage induces Rad9 phosphorylation on multiple [S/T]Q sites, including T603 and other [S/T]Q sites within the Rad9 SCD.

To further directly identify Rad9 phosphorylation sites, we generated and purified antibodies that specifically recognize phosphorylated Rad9 T390, the first [S/T]Q site within the SCD. This site is preceded by a serine and thus is not predicted to be strongly recognized by the phospho-[S/T]Q antibody. Anti-phospho-T390 recognized the slowest mobility form of phosphorylated Rad9 induced by DNA damage (Figure 2C, lanes 1–3). Immunoreactivity was abolished by alanine substitution of T390 but unaffected by substitution of other SCD [S/T]Q sites or T603 (Figure 2C, lanes 4–10). Preincubation of the phospho-T390 antibody with phosphorylated-T390 peptide (but not with the nonphosphorylated peptide) completely eliminated the immunoreactivity of phosphorylated Rad9 (data not shown). Thus, Rad9 T390 is another of the Rad9 [S/T]Q sites that are phosphorylated in response to DNA damage.

Redundant Function of Individual [S/T]Q Sites within the Rad9 SCD

We evaluated the effects of single alanine substitutions within the Rad9 SCD to determine whether a single site within the Rad9 SCD has a dominant role in the phosphorylation of Rad9 and the interaction of Rad9 with Rad53. In these and subsequent experiments, activation of the DNA replication checkpoint pathway was avoided

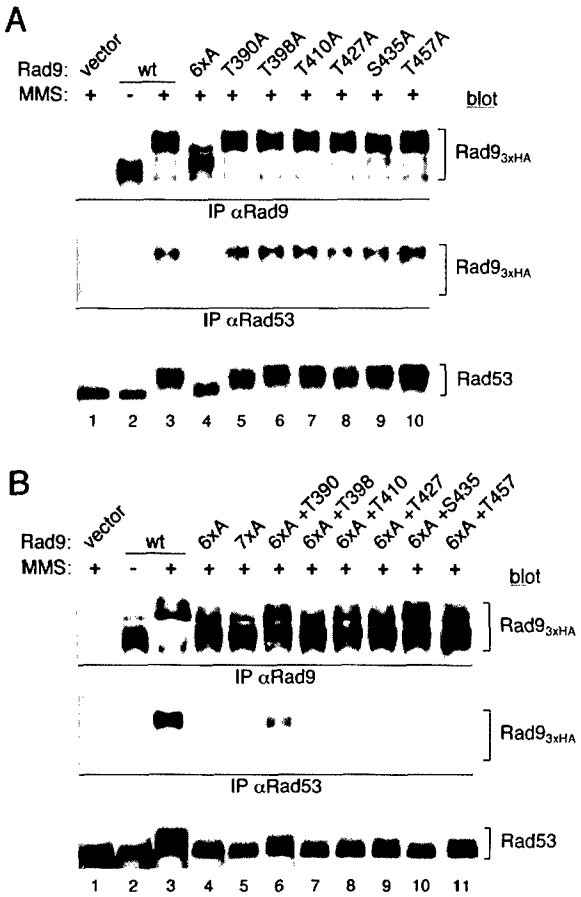


Figure 3. The Rad9 SCD Is Required for the Phosphorylation and Interaction of Rad9 and Rad53

In (A) and (B), anti-HA (top and middle panels) and anti-Rad53 (bottom panel) immunoblot analyses of anti-Rad9 (top panel) and anti-Rad53 (middle panel) immunoprecipitates and corresponding lysates (bottom panel) from the indicated *RAD9* or *rad9* strains. Cells were synchronized in telophase at the *cdc15-2* arrest point and either mock treated (−) or treated with 0.1% MMS (+) for 1 hr. The DLY418-derived strains carry a plasmid encoding *RAD9*, *rad9*^{6xA}, (A) alanine substitutions of individual [S/T]Q sites within the Rad9 SCD, or (B) *rad9*^{7xA}, and *rad9*^{6xA} strains with single wild-type residues restored.

by synchronization of cells in telophase using the conditional *cdc15-2* allele (Futcher, 1999), thus allowing evaluation of Rad53 phosphorylation solely dependent on *RAD9*. Similar to the alanine substitution of Rad9 T603, substitution of single [S/T]Q sites within the SCD had little impact on the DNA damage-induced phosphorylation of Rad9, coimmunoprecipitation of Rad9 with Rad53, or Rad53 phosphorylation (Figure 3A). Single mutation of the Rad9 SCD sites also had little impact on the phospho-[S/T]Q immunoreactivity of phosphorylated Rad9 (data not shown). Since simultaneous substitution of all six SCD sites does inactivate these functions (Figure 3A, lane 4), these data suggest that multiple SCD sites are phosphorylated and that Rad53 interacts redundantly with some or all of these sites.

Rad53 specifically interacts with phosphorylated Rad9 (Sun et al., 1998; Emili, 1998). To characterize the ability of single Rad9 SCD sites to support this interaction, we restored single [S/T]Q residues in the Rad9^{6xA} SCD. Reintroduction of a wild-type residue at Rad9 S435 best, although incompletely, restored the DNA damage-induced slower mobility forms of Rad9 but only modestly rescued coimmunoprecipitation with Rad53 and Rad53 phosphorylation (Figure 3B, lane 10). By contrast, reintroduction of Rad9 T390 moderately restored the DNA damage-induced slower mobility forms and also the interaction of Rad9 and Rad53 (Figure 3B, lane 6). No single Rad9 cluster add-back allele fully restored the checkpoint-induced phosphorylation or coimmunoprecipitation of Rad9 and Rad53. Thus, multiple Rad9 SCD phosphorylation sites act in an additive or cooperative manner to recruit Rad53.

Rad53 FHA Domains Bind Phosphorylated Rad9 T390 In Vitro

The ability of Rad9^{6xA} T390 to restore a significant proportion of the Rad9 interaction with Rad53 implies that T390 is a major in vivo functional site for Rad53 binding. To confirm that Rad53 binds directly to Rad9 T390 in a phosphorylation-dependent manner, we measured the increase of surface plasmon resonance (SPR) caused by binding of soluble, bacterially produced Rad53 FHA domains to synthetic Rad9 T390 peptides immobilized on a BIAcore sensor chip. Rad53 FHA1 specifically interacted with the phosphorylated Rad9 T390 peptide (*P*-T390) (Figure 4A, middle versus left panel). Mutation of two conserved FHA domain residues abolished the binding of the Rad53 FHA1 protein to the phosphorylated T390 peptide (Figure 4A, right panel). Similar to FHA1, GST-Rad53 FHA2 preferentially bound the phosphorylated Rad9 T390 (Figure 4B, middle versus left panel), demonstrating an affinity lost upon substitution of conserved FHA2 residues (Figure 4B, right panel). On average, Rad53 FHA1 bound the phosphorylated Rad9 T390 peptide with an apparent K_D of 2.5 μM (s = 0.3, n = six experiments), and GST-Rad53 FHA2 bound with an apparent K_D of 1.4 μM (s = 0.3, n = three experiments). These affinities are within the general range of those reported for FHA domain-phosphopeptide binding (Durocher et al., 1999; Li et al., 1999; Liao et al., 1999, 2000; Durocher et al., 2000; Wang et al., 2000; Byeon et al., 2001). However, the observed affinity of GST-FHA2 may have been artificially increased due to the ability of GST to homodimerize (Ladbury et al., 1995). In addition, SPR-based determination of FHA domain binding affinity has been demonstrated to overestimate K_D by as much as 20-fold, as compared to binding affinity determined by isothermal calorimetry (Durocher et al., 1999).

In a filter binding assay with immobilized synthetic peptides, GST-Rad53 FHA2 also preferentially bound the phosphorylated form of a Rad9 T603 peptide (data not shown). In a recent study of Rad53 FHA2 binding Rad9 phosphothreonine peptides that fit a Txx[I/L] consensus, Rad53 FHA2 bound a phospho-T603 peptide with a K_D of 12.9 μM (Byeon et al., 2001). Taken together, these results show that the Rad53 FHA domains can directly and specifically bind Rad9 phosphopeptides containing physiological PIKK phosphorylation sites.

Rad9 Phosphorylation Sites Are Required for the Survival of Genotoxic Stress

Survival of genotoxic stress requires the activities of the DNA checkpoint pathways, as well as the DNA repair

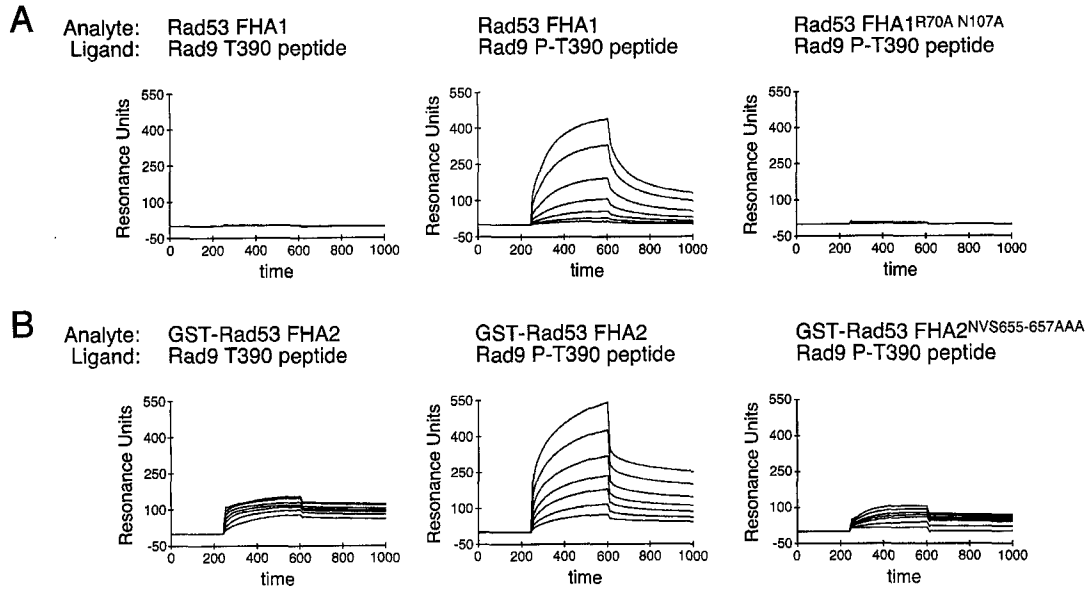


Figure 4. Phosphorylation-Dependent Binding of Rad53 FHA Domains to Rad9 T390 In Vitro

Synthetic peptides containing Rad9 amino acids 382–397, either nonphosphorylated (T390) or phosphorylated (*P*-T390) at T390, were biotinylated and coated onto a BIAcore sensor chip. The sensorgrams indicate binding measured by an increase in surface plasmon resonance during analyte flow over the chip surface, with the nonspecific signal from the biotin-only surface subtracted.

(A) Concentrations of 0.1–4.8 μM of bacterially produced Rad53 FHA1 (left and middle) or Rad53 FHA1 with mutations in conserved FHA domain residues (right) were flowed across the nonphosphorylated (left) and phosphorylated (middle and right) Rad9 T390 peptide surfaces.

(B) Concentrations of 0.19–4.9 μM of bacterially produced GST-Rad53 FHA2 (left and middle) or GST-Rad53 FHA2 with mutations in conserved FHA domain residues (right) were flowed across the nonphosphorylated (left) and phosphorylated (middle and right) Rad9 T390 peptide surfaces. The low level of peptide- and FHA domain-independent resonance observed in (B) was specific to that sensor chip, since it was not seen when the same GST-FHA2 preparation was flowed over the sensor chip used in (A).

systems. Rad9 is involved in both signaling through the DNA damage checkpoint pathway and DNA repair, such that loss of *RAD9* decreases the viability of cells challenged with DNA damage (Weinert and Hartwell, 1988, 1990; Terleth et al., 1990; de la Torre-Ruiz and Lowndes, 2000). Therefore, we determined whether DNA damage-induced Rad9 phosphorylation sites are required for Rad9 function in response to a variety of genotoxic stresses.

Cdc13 is a telomere-associated protein required for the maintenance of chromosome ends (Garvik et al., 1995; Nugent et al., 1996; Lin and Zakian, 1996). At elevated temperatures, cells bearing the temperature-sensitive *cdc13-1* allele exhibit a rapid loss of viability (Weinert et al., 1994). Cells bearing *rad9*^{7xA} were compromised for survival at 37°C in a *cdc13-1* background, nearly as severely as a *rad9*Δ strain (Figure 5A). The *cdc13-1 rad9*^{6xA} strain demonstrated an intermediate loss of viability, suggesting that Rad9 T603 supports a moderate level of Rad9 function in vivo. Consistent with their partial recovery of Rad53 phosphorylation, *cdc13-1 rad9*$^{6xA+T390}$ cells are more viable at 37°C than the *cdc13-1 rad9*^{6xA} strain (Figure 5A).

The absence of Rad9 phosphorylation sites in *rad9*^{7xA} cells also reduced survival of UV irradiation, but this loss of viability was less than that associated with the loss of *RAD9* (Figure 5B, right panels). Interestingly, the *rad9*^{7xA} strain demonstrated a loss of viability approaching that of a *rad9*Δ strain when grown on media containing MMS (Figure 5B, left panels), suggesting that the Rad9 [S/T]Q phosphorylation sites play a greater role in the *RAD9*-dependent survival of MMS-derived DNA damage than in the survival of UV irradiation.

Survival of replication block due to hydroxyurea (HU) treatment is normally *RAD9* independent and involves the activation of Rad53 through the DNA replication checkpoint pathway (Weinert et al., 1994; Allen et al., 1994; Navas et al., 1996). However, if the replication checkpoint pathway is compromised, such as in a *tof1*Δ mutant, Rad53 regulation and HU survival involves signaling through Rad9 (Navas et al., 1996; Foss, 2001). Therefore, we determined the effect of the loss of Rad9 [S/T]Q phosphorylation sites on HU survival in a *tof1*Δ strain. As expected, wild-type, *rad9*Δ, and *rad9*^{7xA} strains were similarly viable when grown on HU-containing media (Figure 5C). The *tof1*Δ mutant was slightly impaired for viability, and the *tof1*Δ *rad9*Δ double mutant was very sensitive to HU (Figure 5C). The *tof1*Δ *rad9*^{7xA} strain was nearly as sensitive as the *tof1*Δ *rad9*Δ strain, indicating a strong requirement for Rad9 [S/T]Q phosphorylation sites for the survival of HU treatment when the replication checkpoint pathway is impaired.

G_2/M DNA Damage Checkpoint Arrest in Rad9 [S/T]Q Mutants

At elevated temperatures, *cdc13-1* strains accumulate telomeric single-stranded DNA that induces a strong *RAD9*-dependent cell cycle arrest at G_2/M (Weinert and Hartwell, 1993; Lydall and Weinert, 1995; Garvik et al., 1995). Rad53 and Chk1 act in parallel downstream of Rad9 to enforce this arrest, such that *rad53* or *chk1* cells have a partial delay and are unable to maintain the

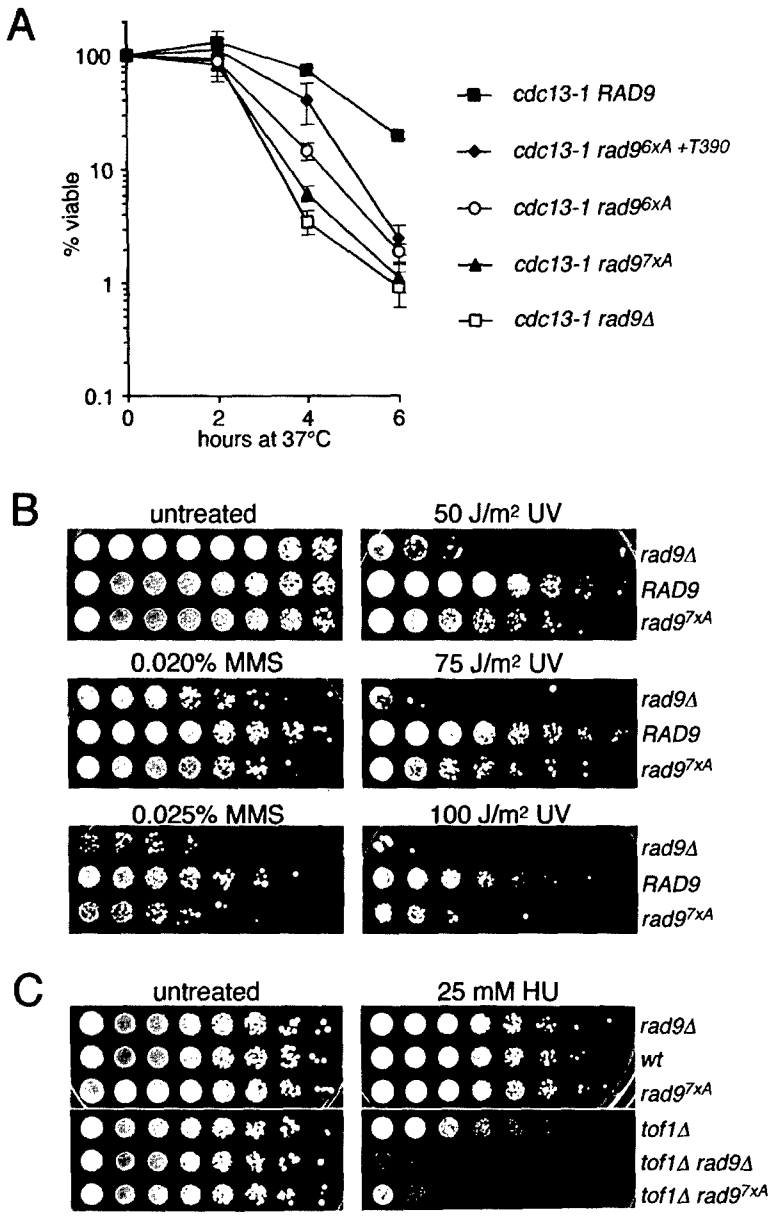


Figure 5. Genotoxin Sensitivity of *rad9* [S/T]Q Mutants

(A) Log-phase cultures of 1588-4C-derived *cdc13-1* strains expressing the indicated forms of Rad9 were shifted to 37°C, and their viability was measured as the number of colony-forming units when plated at permissive temperature. Error bars indicate ± the standard deviation between two matched strains. (B and C) Serial 4-fold dilutions of the (B) 1588-4C-derived *rad9Δ* or (C) U960-5C-derived *rad9Δ* and *rad9Δ tof1Δ* strains carrying plasmids to generate the illustrated genotypes were spotted onto plates either untreated, UV irradiated, containing MMS, or containing HU as indicated. The lethality of *rad53Δ* in U960-5C-derived strains is suppressed by *sml1-1*.

G_2/M cell cycle arrest (Sun et al., 1998; Gardner et al., 1999; Sanchez et al., 1999; Liu et al., 2000). To determine the relationship of Rad9 phosphorylation sites required for Rad53 activation with the functionality of the DNA damage checkpoint pathway, we ascertained the effect of Rad9 [S/T]Q mutations on the G_2/M arrest in *cdc13-1* cells. Similar to *rad53* cells, *rad9*^{6xA} and *rad9*^{7xA} cells transiently delayed prior to anaphase, but eventually escaped into telophase (Figure 6A). The *rad9*^{7xA} defect was consistently slightly more severe than the *rad9*^{6xA} defect, demonstrating that Rad9 T603 partially contributes to the function of Rad9 in maintaining the G_2/M DNA damage checkpoint arrest. Consistent with the ability of the T390 add-back mutant to significantly restore the DNA damage-induced interaction of Rad9 with Rad53, reintroduction of Rad9 T390, or, less effectively, S435, to *rad9*^{6xA} partially restored G_2/M arrest (Figure 6B). These rescues were intermediate, suggesting that the regulation of Rad53 by multiply phosphorylated Rad9 is required for the full action of the *RAD53*-dependent G_2/M DNA damage checkpoint.

Rad9 [S/T]Q Mutants Retain Regulation of Chk1

RAD9 is required for the DNA damage-induced phosphorylation of Chk1, and Rad9 interacts with Chk1 in a yeast two-hybrid assay (Sanchez et al., 1999), suggesting that Rad9 may also directly recruit Chk1 to the DNA damage checkpoint pathway. Therefore, we determined whether the DNA damage-induced phosphorylation of Chk1 is intact in *rad9*^{7xA} cells. As expected, the DNA damage-dependent phosphorylation of Rad53 and Chk1 was absent in a *rad9Δ* strain, and Rad53 phosphorylation was abrogated in *rad9*^{7xA} cells (Figure 7). However, the damage-dependent phosphorylation of Chk1 was not lost in the *rad9*^{7xA} strain (Figure 7, lanes 5–6). These data indicate that the major DNA damage-induced Rad9 phosphorylation sites are not essential for the regulation of Chk1 by Rad9.

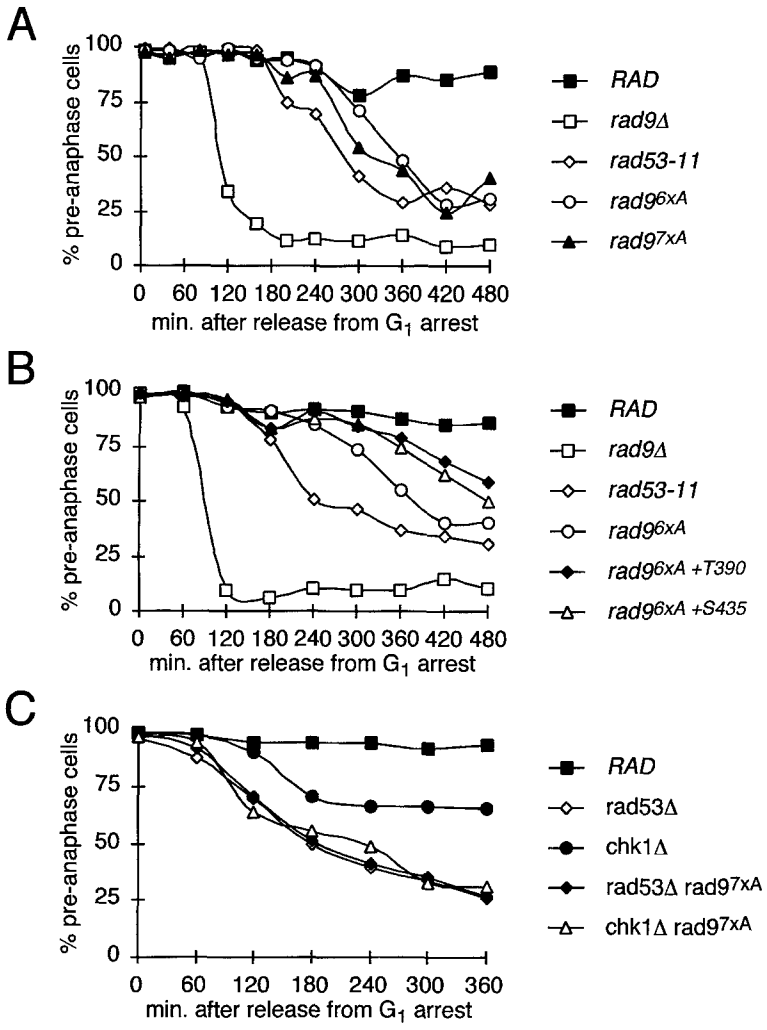


Figure 6. Rad9 Phosphorylation Sites Are Required for the G_2/M DNA Damage Checkpoint Arrest

The ability of the DNA damage checkpoint pathway to enforce the G_2/M arrest as pre-anaphase cell in the face of DNA damage from *cdc13-1* was assessed in the indicated DLY408-derived strains.

(A) Comparison of Rad9 phosphorylation site mutants to wild-type, *rad9*Δ, and *rad53-11*.

(B) Comparison of Rad9 SCD mutants bearing single intact SCD sites at T390 or S435 to *rad9*^{6xA}.

(C) Comparison of *rad53*Δ or *chk1*Δ to the double *rad53*Δ *rad9*^{7xA} or *chk1*Δ *rad9*^{7xA} mutants. The lethality of *rad53*Δ in these cells was suppressed by deletion of *SML1*.

Rad53 and Chk1 both act to enforce the G_2/M checkpoint arrest (Sun et al., 1998; Gardner et al., 1999; Sanchez et al., 1999; Liu et al., 2000). Thus, the intermediate G_2/M checkpoint arrest defect in the *rad9*^{7xA} cells (Figure 6A) could be due to disruption of either Rad53 or Chk1 function. To confirm that the G_2/M arrest defect in the *rad9*^{7xA} cells reflects the failure of the DNA damage checkpoint pathway in coupling to Rad53 rather than Chk1, we determined the effect of the rad9^{7xA} mutation in *chk1*Δ and *rad53*Δ cells. *rad53*Δ *rad9*^{7xA} cells were as defective in the G_2/M DNA damage checkpoint arrest as *rad53*Δ cells, suggesting that the two mutations disrupt the same pathway (Figure 6C). By contrast, *chk1*Δ *rad9*^{7xA} cells were less competent for the function of the G_2/M checkpoint arrest than *chk1*Δ cells (Figure 6C), suggesting that Chk1 and the Rad9 phosphorylation sites function in separate pathways. This is consistent with the observation that loss of the Rad9 phosphorylation sites disrupts the *RAD9*-dependent phosphorylation of Rad53 and not Chk1.

Discussion

Site-directed mutagenesis identified a requirement for Rad9 [S/T]Q sites in the DNA damage-induced Rad9 phosphorylation and interaction with Rad53. Western blot analysis with phosphorylation-specific antibodies directly implicated specific Rad9 [S/T]Q residues as physiological DNA damage-induced phosphorylation sites, and these Rad9 [S/T]Q phosphopeptides are bound by Rad53 FHA domains in vitro. Rad9 phosphorylation site mutants are partially defective for genotoxin survival and function of the G_2/M checkpoint arrest in a manner consistent with dysfunction of the Rad53 effector pathway. These defects caused by Rad9 mutation are specific for its role in Rad53 regulation, since they have little effect on the phosphorylation and function of Chk1.

Rad53 FHA Domain Binding Sites within Rad9

Prior analyses of the phosphopeptide binding properties of FHA domains were performed exclusively in vitro using phosphopeptides of unknown physiological relevance (Durocher et al., 1999, 2000; Liao et al., 2000; Byeon et al., 2001). Studies of Rad53 FHA domains binding to peptide libraries determined that Rad53 FHA1 prefers phosphothreonine followed by aspartate at the +3 position, while Rad53 FHA2 prefers phosphothreonine followed by isoleucine or leucine at +3 (Durocher et al., 1999, 2000; Liao et al., 2000; Byeon et al., 2001). Rad9 T390, the individual Rad9 SCD site which best supports the Rad9 interaction with Rad53 in vivo,

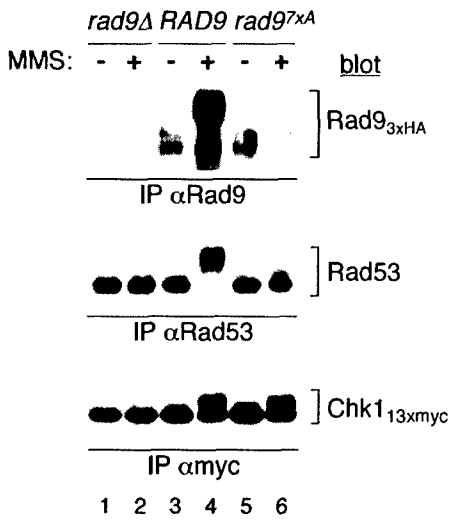


Figure 7. Chk1 Phosphorylation Is Not Disrupted in a Rad9 SCD Mutant

Immunoprecipitation and immunoblot analyses of anti-Rad9 (top), anti-Rad53 (middle), and anti-myc (bottom) from DLY418-derived strains expressing the indicated forms of Rad9. Cells were synchronized in telophase at the *cdc15-2* arrest point and either mock treated (−) or treated with 0.1% MMS (+) for 1 hr.

is followed by valine at +3 and thus precisely fits neither of these preferred patterns. However, both Rad53 FHA domains have a weaker general selectivity for a hydrophobic residue at +3 (Durocher et al., 2000; Byeon et al., 2001), which is consistent with Rad9 T390. Four of the five TQ sites within the Rad9 SCD and six of the nine total TQ sites within Rad9, including T603, have isoleucine, leucine, or valine residues at the +3 position. The identification of phosphorylated Rad9 [S/T]Q sites as in vivo binding targets for the Rad53 FHA domains is in agreement with the prediction that Mec1/Tel1 phosphorylation sites within Rad9 are required for its interaction with Rad53.

Rad9 [S/T]Q Phosphorylation Sites Regulate Rad53 Activation

Single mutation of Rad9 [S/T]Q sites did not have a significant effect on the mobility shift of phosphorylated Rad9 or on the interaction of phosphorylated Rad9 with Rad53. Multiple substitutions of these sites, however, revealed their requirement for Rad9 phosphorylation and regulation of Rad53. Moreover, individual sites within the Rad9 SCD are able to support partial levels of Rad9 phosphorylation and interaction with Rad53, but no single site accounts for all of either function. These data imply that multiple Rad9 [S/T]Q phosphorylation sites together mediate the interaction of Rad9 with Rad53.

The CDK inhibitor Sic1 is phosphorylated at multiple CDK consensus sites. Individually, these sites constitute only moderate-affinity Cdc4 binding sites, but these phosphorylation sites act in concert to target Sic1 for Cdc4-mediated degradation once a threshold of Sic1 phosphorylation is achieved (Nash et al., 2001). Similarly, multiple phosphorylation of [S/T]Q sites within Rad9 may together act to achieve a threshold of Rad53 binding required to induce its phosphorylation and activation. This activation could be achieved both through the transphosphorylation of Rad53 by Mec1/Tel1 and through the concentration-dependent transphosphorylation of Rad53 by Rad53 (Gilbert et al., 2001).

Rad53 is itself potentially bivalent for interactions with Rad9, since both Rad53 FHA domains bind phosphorylated Rad9 (Sun et al., 1998; Durocher et al., 1999), and both FHA domains bind Rad9 phosphopeptides in vitro. Hence, multivalent interactions between multiply phosphorylated Rad9 and bivalent Rad53 may stabilize the Rad9-Rad53 interaction. The contact of an FHA domain to a phosphopeptide directly involves at least six residues encompassing the phosphorylation site (Durocher et al., 2000). Considering that the six Rad9 SCD sites are spread over 69 amino acids, a single Rad9 SCD may engage with multiple Rad53 FHA domains. Furthermore, both Rad9 (Soulier and Lowndes, 1999) and Rad53 (Z.S. and D.F.S., unpublished data) are capable of homotypic interactions. Taken together, these intricate interactions may contribute to aggregation of oligomeric checkpoint complexes. Through the accumulation of these complexes, the ability of Rad53 and similarly its mammalian homolog, Chk2, to self-associate and activate may accelerate a positive feedback loop promoting the activation of DNA damage checkpoint effectors (Gilbert et al., 2001; Xu et al., 2002; Ahn et al., 2002).

Rad9 participates in a number of DNA damage checkpoint pathway-mediated responses to DNA damage (reviewed in Lowndes and Murguia, 2000), including the regulation of Chk1 phosphorylation (Sanchez et al., 1999). Mutation of the Rad9 [S/T]Q phosphorylation sites does not abolish the phosphorylation or function of Chk1. Thus, the domains within Rad9 essential for the regulation of Rad53 and Chk1 are functionally separable, and the mechanism of regulation of Chk1 by Rad9 remains to be determined. The fact that the major damage-induced Rad9 phosphorylation sites are dispensable for the regulation of Chk1 correlates with the lack of recognizable phosphorylation-dependent interaction domains, such as an FHA domain, within Chk1.

SCDs as Targets of DNA Checkpoint Phosphorylation and Interaction

Individual SQ or TQ dipeptides are fairly common in polypeptide sequences, theoretically occurring approximately once every 200 or so residues. However, clusters of [S/T]Q sites are readily recognizable in a handful of known DNA checkpoint or replication proteins. A pattern-based search for SCD of at least five [S/T]Q sites separated by no more than 35 amino acids identified roughly 1% of the human, murine, or budding yeast PROSITE database. Of these, 10% are readily recognizable as DNA checkpoint proteins, including human and/or murine Atm, DNA-PKcs, Brca1, Brca2, Chk2, and Mdm2, and yeast Rad9, Rad53, and Chk1. Experimental evidence already suggests that the SCDs in Brca1, Chk2, and Mdm2 are targets for phosphorylation by PIKKs (Cortez et al., 1999; Sanchez et al., 1999; de Toledo et al., 2000; Matsuoka et al., 2000; Melchionna et al., 2000; Wakayama et al., 2001). Within the group of proteins identified by the PROSITE pattern search, the mammalian DNA-PKcs and yeast Rad9, Rad53, and

Chk1 SCDs are notably rich in TQ residues. Since FHA domains have a strong binding preference for phosphothreonine over phosphoserine (Durocher et al., 1999) and since the threonine-rich Rad9 SCD is a binding site for Rad53 FHA domains, TQ-rich clusters may often be PIKK-dependent FHA domain binding sites. It remains to be determined whether phosphorylated SCDs in proteins other than Rad9 similarly mediate FHA domain interactions or serve other regulatory functions.

Interactions between phosphorylated Rad9 and Rad53 are a prototype for assembly of DNA damage-dependent checkpoint complexes. Two important, unanticipated features of these complexes are the involvement of an intermediary adaptor protein for functional connection of a PIKK with a substrate and the PIKK phosphorylation dependence of that interaction. These features, and the potential multivalency of these interactions, may be common vehicles for the rapid assembly of focal eukaryotic checkpoint complexes at sites of DNA damage.

Experimental Procedures

Plasmids

For pRS306 *rad9*^{T603A} and pRS306 *rad9*$^{\Delta 593-620}$, the mutations were introduced by PCR into the SpeI-HindIII internal *RAD9* fragment cloned into the same sites of pRS306. To construct pRS306 *RAD9*^{3xHA}, the FLAG-tag of pRS314G *RAD9*^{FLAG} (Sun et al., 1998) was replaced with a 3xHA tag followed by ~500 bp of *RAD9* 3′ UTR, and the MunI-SacII fragment was cloned into pRS306. pRS316 *RAD9*^{3xHA} was constructed by replacing the MunI-SacII fragment of pRS316 *RAD9*^{FLAG} (Sun et al., 1998) with the MunI-SacII from pRS306 *RAD9*^{3xHA}. pRS306 *rad9*Δ was created by replacing the *RAD9* coding sequence in the XhoI-SmaI fragment of pRS306 *RAD9*^{3xHA} with ~1.17 kb of *RAD9* 5′ UTR. To create *CHK1*^{13xmyc}, the *CHK1* ORF and surrounding UTR was isolated by PCR and cloned into pBSKII^{+}. The SmaI-SpeI fragment encoding the 13xmyc tag from pFA6a-13myc-His3MX6 (Longtine et al., 1998) was inserted into equivalent sites created by PCR at the 3′ end of the *CHK1* ORF. The entire *CHK1*^{13xmyc} locus was then cloned into pRS315. For the GST-FHA1 expression construct, *RAD53* encoding amino acids 1–197 were amplified by PCR and cloned into pGEX4T3. The alanine substitutions of the conserved FHA domain residues at R70 and N107 were introduced by PCR. The GST-Rad53 FHA2 fusion proteins were described previously (Sun et al., 1998).

Strains

DLY408 (*cdc13-1 cdc15-2*), DLY409 (*cdc13-1 cdc15-2 rad9::HIS3*), DLY418 (*cdc15-2*), and DLY554 (*cdc13-1 cdc15-2 rad53-21*) are from the Weinert laboratory (Gardner et al., 1999; Lydall and Weinert, 1997). 1588-4C and U960-5C (*sml1-1 rad53-XB::HIS3*) are from the Rothstein laboratory (Zhao et al., 1998). These strains are in a W303 background.

The initial *rad9*^{T603A} and *rad9*$^{\Delta 593-620}$ mutations were engineered into DLY408 by two-step allele replacement with the appropriate pRS306 constructs. pRS306 *RAD9*^{3xHA} was used to epitope tag *RAD9* in DLY408. For the site-directed mutagenesis of Rad9 [S/T]Q sites, PCR cassettes containing the different mutations were used to replace the corresponding sequence in a *rad9*$^{\Delta 593-620}$ strain by two-step allele replacement (Erdeniz et al., 1997). pRS306 *rad9*Δ was used to delete *RAD9* in 1588-4C and DLY408, and *cdc13-1* was subsequently introduced into 1588-4C *rad9*Δ with the plasmid pDL420 (Weinert laboratory). To construct DLY408 *rad53*Δ strains, *SML1* was first replaced with a *sml1::TRP1* cassette PCR amplified from U973 (*sml1::TRP1 esr1-1*, Rothstein laboratory). *RAD53* was then replaced with a *rad53::HIS3* PCR cassette that targets the precise removal of the *RAD53* ORF. *CHK1* and *TOF1* ORFs in DLY408 and U960-5C, respectively, were replaced with kan^{R} cassettes PCR amplified from the corresponding deletion strains (Research Genetics).

Drop-out media was purchased from Bufferad. Synthetic and rich media were typically supplemented with adenine to 47.5 mg/l. For immunoblot analyses, cultures were grown to early/mid-log phase. For *cdc15-2* synchronization, early-log cultures were shifted to 37°C for 3 hr. Cultures were mock treated or treated with 0.1% MMS (Sigma) for 1 hr, washed, and either lysed immediately or frozen in liquid nitrogen and stored at −80°C.

Immunoprecipitations and Immunoblotting

Immunoprecipitations and anti-Rad53 Western blotting were as previously described (Sun et al., 1998). In brief, lysates were prepared by mechanical disruption in TG (PBS + 1% Triton X-100, 10% glycerol, and phosphatase and protease inhibitors [Roche and Sigma]). 1–2.5 mg of lysate was used per immunoprecipitation with rabbit anti-Rad53 serum, rabbit anti-Rad9 serum (G. Liu, M.F.S., and D.F.S., unpublished data), mouse anti-myc monoclonal 9E10 (Covance), or control antibodies. TCA lysates were prepared as described (Foiani et al., 1999), with minor volume adjustments. Lysates and immunoprecipitations were resolved on 6% or 7.5% polyacrylamide gels prior to transfer to Immobilon-P (Millipore).

Rad9^{3xHA} was detected in immunoblot analysis with HRP-conjugated rat anti-HA monoclonal 3F10 (Roche), Rad53 with rabbit anti-Rad53 serum or a goat anti-Rad53 antibody (Santa Cruz), and Chk1^{13xmyc} with HRP-conjugated 9E10 (Santa Cruz). The rabbit anti-phospho-[S/T]Q antibody was used as per manufacturer's suggestions (Cell Signaling Technology). Polyclonal rabbit antibody against Rad9 phospho-T390 was generated and purified as described (DiGiovanna et al., 1998) using the T390 phosphopeptide KSNRST*QIVN as antigen and nonphosphorylated and phosphorylated forms of the peptide TENNSNRST*QIVNNPR for purification.

GST Fusion Protein Purification for BIAcore

Bacterial cultures expressing GST-Rad53 and Rad9 fusion proteins were collected by centrifugation and lysed by sonication in TG with 5 mM DTT. Clarified lysates were rotated with glutathione-sepharose beads at 4°C. The beads were then washed in batch format once with >100× bead bed volume lysis buffer and twice with PBS or HS-t (10 mM HEPES [pH 7.6], 150 mM NaCl, 0.005% Tween-20). For the FHA1 fusions, the latter two washes lacked protease inhibitors, and thrombin cleavage was performed as per manufacturer's suggestions (Amersham). Thrombin was removed by incubation with benzamidine-agarose beads (Sigma). The GST-Rad53 FHA2 construct, a noncleavable GST fusion protein, was eluted with glutathione, and the glutathione was removed by dialysis.

Synthetic Peptides

Rad9 peptides were synthesized and purified by the Small Scale Peptide Synthesis facility at the W.M. Keck Biotechnology Resource Center at the Yale School of Medicine.

BIAcore Peptide Binding Assay

The 16-mer Rad9 T390 peptides were combined with biotin-LC-NHS (Pierce) at a molar ratio of 1.5:1 peptide:biotin overnight at 4°C. Remaining NHS groups were blocked with 20 mM Tris. Biotinylated peptides and a similar biotin-only mixture were diluted and flowed over the surface of a streptavidin-coated sensor chip in a BIAcore 2000 as per manufacturer's instructions (BIAcore). The peptide surfaces typically yielded 70–200 RUs over the biotin-only surface. Analytes were dialyzed into HBS-Tw (10 mM HEPES [pH 7.6], 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween-20). For the SPR measurements, 60 μl of various concentrations of analyte at 10 μl/min or 125 μl at 25 μl/min were flowed over each surface at 15°C. Surface regeneration was accomplished with a pulse of 1 M NaCl, a pulse of 2 M $MgCl_2$, and extensive washing with HBS-Tw.

Genotoxin Sensitivity Assays

For the *cdc13-1* assay, log-phase cultures of 1588-4C *cdc13-1 rad9*Δ strains bearing appropriate plasmids were shifted to 37°C, and aliquots plated in duplicate on YPAD at the indicated times. Plates were incubated at 23°C for 3 days. For the remaining assays, cultures of 1588-4C- or U960-5C-derived strains bearing appropriate plasmids were brought to similar densities and serially diluted 4-fold in a 96-well plate. A 48-pin inoculator was used to spot the diluted cultures onto YPAD, YPAD+HU, or YPAD+MMS plates. Sets

of YPAD plates were UV irradiated in a prewarmed Stratalinker (Stratagene). The plate cultures were grown at 30°C for 2–3 days.

Assay for the G_2/M DNA Damage Checkpoint Function

This assay was performed largely as described (Lydall and Weinert, 1995, 1997). In brief, strains derived from DLY408, DLY409, or DLY554 bearing the appropriate plasmids were grown to early-log phase at 23°C in synthetic media, shifted into YPAD, and synchronized with α factor. Cells were washed into prewarmed YPAD and grown at 37°C. 70% ethanol fixed samples were sonicated and spotted onto poly-L-lysine (Sigma)-coated slides, dried, and sealed under a coverslip with DAPI mounting media (Vector Laboratories). For each sample, at least 100 cells were scored. Cells with gross nuclear or morphological defects and cells terminally G_1 arrested as determined by size and morphology were not scored.

Acknowledgments

We are grateful for the assistance of J. Falato and G. Bellinger, and for strains and reagents from the Weinert, Hartwell, Rothstein, and McAlear laboratories. We thank D. Durocher and members of the Stern laboratory for discussions and critical reading of the manuscript. This work was supported by grants from the A-T Children's Project (Z.S. and D.F.S.), USPHS R01CA82257 (D.F.S.), USAMRMC DAMD17-98-1-8272 (J.K.D. and D.F.S.), and NIDDK P01DK55389 (J.S.M. and D.P.). M.F.S. was supported by NIH NRSA T32GM07223 from the NIGMS and by USAMRMC DAMD 17-99-1-9460.

Received: April 30, 2001
Revised: April 12, 2002

References

Abraham, R.T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev. *15*, 2177–2196.

Ahn, J.Y., Li, X., Davis, H.L., and Canman, C.E. (2002). Phosphorylation of threonine 68 promotes oligomerization and autophosphorylation of the Chk2 protein kinase via the forkhead-associated (FHA) domain. J. Biol. Chem., in press.

al-Khodairy, F., Fotou, E., Sheldrick, K.S., Griffiths, D.J., Lehmann, A.R., and Carr, A.M. (1994). Identification and characterization of new elements involved in checkpoint and feedback controls in fission yeast. Mol. Biol. Cell *5*, 147–160.

Allen, J.B., Zhou, Z., Siede, W., Friedberg, E.C., and Elledge, S.J. (1994). The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes Dev. *8*, 2401–2415.

Anderson, C.W., and Lees-Miller, S.P. (1992). The nuclear serine/threonine protein kinase DNA-PK. Crit. Rev. Eukaryot. Gene Expr. *2*, 283–314.

Bell, D.W., Varley, J.M., Szydlo, T.E., Kang, D.H., Wahrer, D.C., Shannon, K.E., Lubratovich, M., Verselis, S.J., Isselbacher, K.J., Fraumeni, J.F., et al. (1999). Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science *286*, 2528–2531.

Bentley, N.J., Holtzman, D.A., Flaggs, G., Keegan, K.S., DeMaggio, A., Ford, J.C., Hoekstra, M., and Carr, A.M. (1996). The *Schizosaccharomyces pombe* rad3 checkpoint gene. EMBO J. *15*, 6641–6651.

Bork, P., Hofmann, K., Bucher, P., Neuwald, A.F., Altschul, S.F., and Koonin, E.V. (1997). A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins. FASEB J. *11*, 68–76.

Byeon, I.J., Yongkiettrakul, S., and Tsai, M.D. (2001). Solution structure of the yeast Rad53 FHA2 complexed with a phosphothreonine peptide pTXXL: comparison with the structures of FHA2-pYXL and FHA1-pTXXD complexes. J. Mol. Biol. *314*, 577–588.

Chehab, N.H., Malikzay, A., Appel, M., and Halazonetis, T.D. (2000). Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes Dev. *14*, 278–288.

Cortez, D., Wang, Y., Qin, J., and Elledge, S.J. (1999). Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. Science *286*, 1162–1166.

de la Torre-Ruiz, M., and Lowndes, N.F. (2000). The *Saccharomyces cerevisiae* DNA damage checkpoint is required for efficient repair of double strand breaks by non-homologous end joining. FEBS Lett. *467*, 311–315.

de Toledo, S.M., Azzam, E.I., Dahlberg, W.K., Gooding, T.B., and Little, J.B. (2000). ATM complexes with HDM2 and promotes its rapid phosphorylation in a p53-independent manner in normal and tumor human cells exposed to ionizing radiation. Oncogene *19*, 6185–6193.

DiGiovanna, M.P., Roussel, R.R., and Stern, D.F. (1998). Production of antibodies that recognize specific Tyrosine-phosphorylated peptides. In Current Protocols in Molecular Biology, F.M. Ausubel, et al., eds. (New York: John Wiley & Sons), pp. 18.6.1–18.6.19.

Durocher, D., and Jackson, S.P. (2001). DNA-PK, ATM and ATR as sensors of DNA damage: variations on a theme? Curr. Opin. Cell Biol. *13*, 225–231.

Durocher, D., Henckel, J., Fersht, A.R., and Jackson, S.P. (1999). The FHA domain is a modular phosphopeptide recognition motif. Mol. Cell *4*, 387–394.

Durocher, D., Taylor, I.A., Sarbassova, D., Haire, L.F., Westcott, S.L., Jackson, S.P., Smerdon, S.J., and Yaffe, M.B. (2000). The molecular basis of FHA domain:phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms. Mol. Cell *6*, 1169–1182.

Elledge, S.J. (1996). Cell cycle checkpoints: preventing an identity crisis. Science *274*, 1664–1672.

Emili, A. (1998). MEC1-dependent phosphorylation of Rad9p in response to DNA damage. Mol. Cell *2*, 183–189.

Erdeniz, N., Mortensen, U.H., and Rothstein, R. (1997). Cloning-free PCR-based allele replacement methods. Genome Res. *7*, 1174–1183.

Foiani, M., Liberi, G., Piatti, S., and Plevani, P. (1999). *Saccharomyces cerevisiae* as a model system to study DNA replication. In Eukaryotic DNA Replication: A Practical Approach, S. Cotterill, ed. (Oxford, UK: Oxford University Press), pp. 185–200.

Foss, E. (2001). Tof1p regulates DNA damage responses during S phase in *Saccharomyces cerevisiae*. Genetics *157*, 567–577.

Futcher, B. (1999). Cell cycle synchronization. Methods Cell Sci. *21*, 79–86.

Gardner, R., Putnam, C.W., and Weinert, T. (1999). RAD53, DUN1 and PDS1 define two parallel G2/M checkpoint pathways in budding yeast. EMBO J. *18*, 3173–3185.

Garvik, B., Carson, M., and Hartwell, L. (1995). Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. Mol. Cell. Biol. *15*, 6128–6138.

Gilbert, C.S., Green, C.M., and Lowndes, N.F. (2001). Budding yeast Rad9 is an ATP-dependent Rad53 activating machine. Mol. Cell *8*, 129–136.

Hofmann, K., and Bucher, P. (1995). The FHA domain: a putative nuclear signalling domain found in protein kinases and transcription factors. Trends Biochem. Sci. *20*, 347–349.

Kim, S.T., Lim, D.S., Canman, C.E., and Kastan, M.B. (1999). Substrate specificities and identification of putative substrates of ATM kinase family members. J. Biol. Chem. *274*, 37538–37543.

Koonin, E.V., Altschul, S.F., and Bork, P. (1996). BRCA1 protein products. Functional motifs. Nat. Genet. *13*, 266–268.

Ladbury, J.E., Lemmon, M.A., Zhou, M., Green, J., Botfield, M.C., and Schlessinger, J. (1995). Measurement of the binding of tyrosyl phosphopeptides to SH2 domains: a reappraisal. Proc. Natl. Acad. Sci. USA *92*, 3199–3203.

Lee, C.H., and Chung, J.H. (2001). The hCds1 (Chk2)-FHA domain is essential for a chain of phosphorylation events on hCds1 that is induced by ionizing radiation. J. Biol. Chem. *276*, 30537–30541.

Li, J., Smith, G.P., and Walker, J.C. (1999). Kinase interaction domain of kinase-associated protein phosphatase, a phosphoprotein-binding domain. Proc. Natl. Acad. Sci. USA *96*, 7821–7826.

Liao, H., Byeon, I.J., and Tsai, M.D. (1999). Structure and function of a new phosphopeptide-binding domain containing the FHA2 of Rad53. J. Mol. Biol. *294*, 1041–1049.

Liao, H., Yuan, C., Su, M.I., Yongkiettrakul, S., Qin, D., Li, H., Byeon, I.J., Pei, D., and Tsai, M.D. (2000). Structure of the FHA1 domain of yeast Rad53 and identification of binding sites for both FHA1 and its target protein Rad9. J. Mol. Biol. *304*, 941–951.

Lin, J.J., and Zakian, V.A. (1996). The Saccharomyces CDC13 protein is a single-strand TG1-3 telomeric DNA-binding protein in vitro that affects telomere behavior in vivo. Proc. Natl. Acad. Sci. USA *93*, 13760–13765.

Lindsay, H.D., Griffiths, D.J., Edwards, R.J., Christensen, P.U., Murray, J.M., Osman, F., Walworth, N., and Carr, A.M. (1998). S-phase-specific activation of Cds1 kinase defines a subpathway of the checkpoint response in *Schizosaccharomyces pombe*. Genes Dev. *12*, 382–395.

Liu, Y., Vidanes, G., Lin, Y.C., Mori, S., and Siede, W. (2000). Characterization of a *Saccharomyces cerevisiae* homologue of *Schizosaccharomyces pombe* Chk1 involved in DNA-damage-induced M-phase arrest. Mol. Gen. Genet. *262*, 1132–1146.

Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast *14*, 953–961.

Lowndes, N.F., and Murguia, J.R. (2000). Sensing and responding to DNA damage. Curr. Opin. Genet. Dev. *10*, 17–25.

Lydall, D., and Weinert, T. (1995). Yeast checkpoint genes in DNA damage processing: implications for repair and arrest. Science *270*, 1488–1491.

Lydall, D., and Weinert, T. (1997). Use of cdc13-1-induced DNA damage to study effects of checkpoint genes on DNA damage processing. Methods Enzymol. *283*, 410–424.

Matsuoka, S., Rotman, G., Ogawa, A., Shiloh, Y., Tamai, K., and Elledge, S.J. (2000). Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. Proc. Natl. Acad. Sci. USA *97*, 10389–10394.

Melchionna, R., Chen, X.B., Blasina, A., and McGowan, C.H. (2000). Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. Nat. Cell Biol. *2*, 762–765.

Nash, P., Tang, X., Orlicky, S., Chen, Q., Gertler, F.B., Mendenhall, M.D., Sicheri, F., Pawson, T., and Tyers, M. (2001). Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. Nature *414*, 514–521.

Navas, T.A., Sanchez, Y., and Elledge, S.J. (1996). RAD9 and DNA polymerase epsilon form parallel sensory branches for transducing the DNA damage checkpoint signal in *Saccharomyces cerevisiae*. Genes Dev. *10*, 2632–2643.

Nugent, C.I., Hughes, T.R., Lue, N.F., and Lundblad, V. (1996). Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. Science *274*, 249–252.

Paulovich, A.G., Margulies, R.U., Garvik, B.M., and Hartwell, L.H. (1997). RAD9, RAD17, and RAD24 are required for S phase regulation in *Saccharomyces cerevisiae* in response to DNA damage. Genetics *145*, 45–62.

Rhind, N., and Russell, P. (2000). Chk1 and Cds1: linchpins of the DNA damage and replication checkpoint pathways. J. Cell Sci. *113*, 3889–3896.

Sanchez, Y., Desany, B.A., Jones, W.J., Liu, Q., Wang, B., and Elledge, S.J. (1996). Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. Science *271*, 357–360.

Sanchez, Y., Bachant, J., Wang, H., Hu, F., Liu, D., Tetzlaff, M., and Elledge, S.J. (1999). Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. Science *286*, 1166–1171.

Siede, W., Friedberg, A.S., and Friedberg, E.C. (1993). RAD9-dependent G1 arrest defines a second checkpoint for damaged DNA in the cell cycle of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *90*, 7985–7989.

Soulier, J., and Lowndes, N.F. (1999). The BRCT domain of the *S. cerevisiae* checkpoint protein Rad9 mediates a Rad9-Rad9 interaction after DNA damage. Curr. Biol. *9*, 551–554.

Stone, J.M., Collinge, M.A., Smith, R.D., Horn, M.A., and Walker, J.C. (1994). Interaction of a protein phosphatase with an Arabidopsis serine-threonine receptor kinase. Science *266*, 793–795.

Sun, Z., Fay, D.S., Marini, F., Foiani, M., and Stern, D.F. (1996). Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways. Genes Dev. *10*, 395–406.

Sun, Z., Hsiao, J., Fay, D.S., and Stern, D.F. (1998). Rad53 FHA domain associated with phosphorylated Rad9 in the DNA damage checkpoint. Science *281*, 272–274.

Terleth, C., Schenk, P., Poot, R., Brouwer, J., and van de Putte, P. (1990). Differential repair of UV damage in rad mutants of *Saccharomyces cerevisiae*: a possible function of G2 arrest upon UV irradiation. Mol. Cell. Biol. *10*, 4678–4684.

Vialard, J.E., Gilbert, C.S., Green, C.M., and Lowndes, N.F. (1998). The budding yeast Rad9 checkpoint protein is subjected to Mec1/Tel1-dependent hyperphosphorylation and interacts with Rad53 after DNA damage. EMBO J. *17*, 5679–5688.

Wakayama, T., Kondo, T., Ando, S., Matsumoto, K., and Sugimoto, K. (2001). Pie1, a protein interacting with Mec1, controls cell growth and checkpoint responses in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *21*, 755–764.

Walworth, N., Davey, S., and Beach, D. (1993). Fission yeast chk1 protein kinase links the rad checkpoint pathway to cdc2. Nature *363*, 368–371.

Wang, P., Byeon, I.J., Liao, H., Beebe, K.D., Yongkiettrakul, S., Pei, D., and Tsai, M.D. (2000). II. Structure and specificity of the interaction between the FHA2 domain of Rad53 and phosphotyrosyl peptides. J. Mol. Biol. *302*, 927–940.

Weinert, T.A., and Hartwell, L.H. (1988). The RAD9 gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. Science *241*, 317–322.

Weinert, T.A., and Hartwell, L.H. (1990). Characterization of RAD9 of *Saccharomyces cerevisiae* and evidence that its function acts posttranslationally in cell cycle arrest after DNA damage. Mol. Cell. Biol. *10*, 6554–6564.

Weinert, T.A., and Hartwell, L.H. (1993). Cell cycle arrest of cdc mutants and specificity of the RAD9 checkpoint. Genetics *134*, 63–80.

Weinert, T.A., Kiser, G.L., and Hartwell, L.H. (1994). Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev. *8*, 652–665.

Xu, X., Tsvetkov, L.M., and Stern, D.F. (2002). Chk2 activation and phosphorylation-dependent oligomerization. Mol. Cell. Biol. *21*, 4419–4432.

Zhao, X., Muller, E.G., and Rothstein, R. (1998). A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. Mol. Cell *2*, 329–340.

Zhou, B.B., and Elledge, S.J. (2000). The DNA damage response: putting checkpoints in perspective. Nature *408*, 433–439.

MOLECULAR AND CELLULAR BIOLOGY, June 2002, p. 4419–4432 Vol. 22, No. 12
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.12.4419–4432.2002

Chk2 Activation and Phosphorylation-Dependent Oligomerization

Xingzhi Xu, Lyuben M. Tsvetkov, and David F. Stern*

Department of Pathology, School of Medicine, Yale University, New Haven, Connecticut 06510

Received 17 September 2001/Returned for modification 29 October 2001/Accepted 21 March 2002

The tumor suppressor gene *CHK2* encodes a versatile effector serine/threonine kinase involved in responses to DNA damage. Chk2 has an amino-terminal SQ/TQ cluster domain (SCD), followed by a forkhead-associated (FHA) domain and a carboxyl-terminal kinase catalytic domain. Mutations in the SCD or FHA domain impair Chk2 checkpoint function. We show here that autophosphorylation of Chk2 produced in a cell-free system requires *trans* phosphorylation by a wortmannin-sensitive kinase, probably ATM or ATR. Both SQ/TQ sites and non-SQ/TQ sites within the Chk2 SCD can be phosphorylated by active Chk2. Amino acid substitutions in the SCD and the FHA domain impair auto- and *trans*-kinase activities of Chk2. Chk2 forms oligomers that minimally require the FHA domain of one Chk2 molecule and the SCD within another Chk2 molecule. Chk2 oligomerization in vivo increases after DNA damage, and when damage is induced by gamma irradiation, this increase requires *ATM*. Chk2 oligomerization is phosphorylation dependent and can occur in the absence of other eukaryotic proteins. Chk2 can cross-phosphorylate another Chk2 molecule in an oligomeric complex. Induced oligomerization of a Chk2 chimera in vivo concomitant with limited DNA damage augments Chk2 kinase activity. These results suggest that Chk2 oligomerization regulates Chk2 activation, signal amplification, and transduction in DNA damage checkpoint pathways.

DNA damage activates response pathways that halt the cell cycle, induce the transcription of genes that facilitate DNA repair and DNA replication, alter telomeres, and induce apoptosis if damage cannot be repaired (65). Checkpoint defects may result in genomic instability, a mutagenic condition that predisposes organisms to cancer. On the other hand, DNA-damaging agents, in the form of gamma irradiation and genotoxic drugs, are mainstays of current cancer treatment regimens. Manipulation of checkpoint genes may ultimately benefit chemo- and radiotherapy (23).

Checkpoint pathways are analogous to growth factor-regulated signal transduction pathways, in which DNA damage initiates a signal that is transduced and amplified to generate checkpoint responses. Although the precise nature of the initial step of signal transduction is poorly understood, damaged DNA activates a cascade of protein kinases. In mammals, these kinases include the phosphoinositide kinase-related kinases (PIKKs) Atm (ataxia telangiectasia mutated) and Atr (Atm and Rad3 related) and the downstream serine/threonine checkpoint kinases Chk1 and Chk2. Orthologs of these genes have been identified in yeasts, with *Saccharomyces cerevisiae* Mec1 and Tel1 serving Atm- or Atr-like functions and Chk1 and Rad53 resembling mammalian Chk1 and Chk2, respectively. Effectors that execute the functions of the DNA damage responses include substrates of both PIKKs and checkpoint kinases.

Atm is a central signaling protein in the response to ionizing radiation and other sources of double-strand DNA breaks. Homozygous mutations in *ATM* are responsible for the pleiotropic ataxia-telangiectasia syndrome, which includes cancer predisposition and sensitivity to ionizing radiation along with progressive cerebellar defects (29). Chk2 is a major effector of Atm (6, 7, 10, 40). Both the breast cancer susceptibility gene product Brca1 (16, 33) and p53 (3, 8, 11, 12, 25, 52) are substrates of Atm and Chk2. Li-Fraumeni syndrome is a hereditary disorder predisposing to multiple neoplasms and is generally associated with a constitutional *TP53* mutation. *CHK2* mutations have been identified in some Li-Fraumeni syndrome kindreds that do not have p53 mutations (5, 58), in myelodysplastic syndromes and acute myeloid leukemias (27), in lung cancer (24, 44), in osteosarcoma (44), and in ovarian cancer (44). *CHK2*, therefore, is a regulator of tumor suppressor gene products and is itself a likely tumor suppressor gene.

Chk2 activation in response to ionizing irradiation is *ATM* dependent (6, 7, 10, 40). Activated Chk2 in turn phosphorylates p53 at serine-20 (11, 25, 52), Cdc25A at serine-123 (21), and Cdc25C at serine-216 (6, 7, 10, 40), contributing to the G_1/S, S, and G_2/M checkpoints, respectively.

Atm-like PIKKs show a strong sequence preference for phosphorylation of SQ/TQ sites (2, 30). The SQ/TQ cluster domain (SCD), near the amino terminus of Chk2, includes seven SQ/TQ sites, including known *ATM*- and *ATR*-dependent phosphorylation targets (1, 41, 42, 64). The SCD is followed by a forkhead-associated (FHA) domain and a carboxyl-terminal kinase catalytic domain. Activation of Chk2 occurs through a series of steps, including *trans* phosphorylation by Atm or Atr at sites within the SCD, including T68 (1, 41, 42, 64). PIKK-dependent phosphorylation is required for autophosphorylation within the activation loop of the catalytic domain at T383 and/or T387 (32).

The intact FHA domain is required for damage-dependent activation of Chk2 (11, 32). FHA protein homology domains were first identified in a subset of forkhead transcription factors (26). They are present in a wide variety of proteins in prokaryotes and eukaryotes (34). Many eukaryotic FHA domain-containing proteins are found in the nucleus and are involved in DNA repair and cell cycle arrest (34). Recent work

* Corresponding author. Mailing address: Department of Pathology, School of Medicine, Yale University, 310 Cedar Street, BML 342, New Haven, CT 06510. Phone: (203) 785-4832. Fax: (203)785-7467. E-mail: Df.stern@yale.edu.

shows that FHA domains are phosphopeptide recognition domains (18, 19, 36, 37, 55, 57), and structurally they have some similarity to the MH2 domains of R-Smads (19), signal transducers and transcriptional comodulators of transforming growth factor beta (TGF-β) signaling (39). However, only a small number of protein-protein interactions that are mediated by FHA domains have been identified. They include association of an *Arabidopsis thaliana* phosphatase FHA domain with a phosphorylated receptor-like kinase (35, 55) and interaction of *S. cerevisiae* Rad53 with phosphorylated Rad9 (57), which operates upstream from Rad53 in the damage-dependent signaling cascade.

The FHA domains of the *S. cerevisiae* Chk2 homolog Rad53 couple Rad53 to damage-dependent signals through direct binding to a second damage response protein, Rad9 (57). DNA damage induces PIKK-dependent phosphorylation of Rad9 (20, 47, 56, 59). Once phosphorylated, Rad9 binds tightly to the two FHA domains of Rad53 (18, 36, 37, 57). Disruption of this interaction either through mutations of Rad53 FHA domains (57) or mutations in the Rad9 sites that bind Rad53 FHA domains (49a) prevents activation of Rad53. Since Rad9 and Rad53 both require Mec1 for activating phosphorylations, these results suggested that phospho-Rad9 acts as an adaptor that recruits Rad53 to an activating complex containing Mec1. Alternatively, it has been proposed that phosphorylated Rad9 dimer functions as a scaffold to bring Rad53 molecules into close proximity to each other, facilitating cross-phosphorylation between Rad53 molecules and subsequent release of activated Rad53 (22).

Activation of protein kinases through regulated oligomerization has been demonstrated for both tyrosine kinases and serine/threonine kinases (28, 38, 50). We report here that Chk2 undergoes oligomerization in response to DNA damage. This process is mediated by the phosphorylated SCD in an activated Chk2 molecule and the FHA domain in another Chk2 molecule. With limited DNA damage, oligomerization of Chk2 modulates phosphorylation and kinase activity. We propose that Chk2 oligomerization is central to regulation of Chk2 activation, signal transduction, and signal amplification.

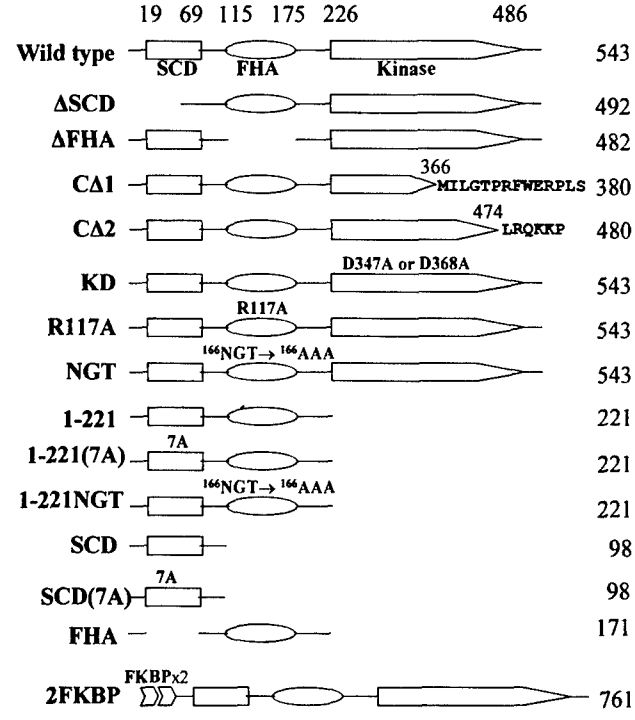


FIG. 1. Schematic diagrams of Chk2 and Chk2 mutants. SCD, FHA, and kinase catalytic domains are marked, with amino acid coordinates above. CΔ1 corresponds to a spontaneous variant of *CHK2* from Li-Fraumeni syndrome, with a frameshift mutation causing readthrough into alternate reading frames as indicated. CΔ2, a similar frameshift mutation thought to be a spontaneous variant of *CHK2* from Li-Fraumeni syndrome, was found to be a polymorphism in the homologous fragment present on chromosome 15 (53). KD is kinase defective owing to substitutions of conserved residues in the catalytic domain. R117A and NGT/AAA are substitutions of conserved FHA domain residues. 7A has alanine substituted for each S or T of all seven SQ and TQ sites within the SCD. 2FKBP contains two copies of the FKBP mutant (F36V) fused to the amino terminus of Chk2.

MATERIALS AND METHODS

Antibodies. Rabbit polyclonal anti-Chk2 T26/S28 was a kind gift from Yi Tan (Cell Signaling Technology). This antibody recognizes Chk2 after gamma irradiation, but not when T26 and S28 have been replaced with alanine (X. Xu and D. F. Stern, unpublished data). Rabbit anti-phospho-T68 was produced by immunization with keyhole limpet hemocyanin coupled to KSSLETVSpTQELYSI, where pT is phosphothreonine. This antibody recognizes Chk2 after gamma irradiation, but not when T68 has been replaced with alanine (Fig. 7E and data not shown). Mouse monoclonal anti-HA antibody (16B12) was purchased from Covance; mouse monoclonal anti-Flag, rabbit anti-glutathione-*S*-transferase (GST), and mouse immunoglobulin G (IgG) were purchased from Sigma; horseradish peroxidase-conjugated mouse antihemagglutinin (HA) (12CA5) and rat anti-HA (3F10) monoclonal antibodies were from Roche; and goat anti-Chk2 (N-17) was from Santa Cruz. Antigen-antibody complexes were recovered with protein G plus protein A-agarose (CalBiochem). Horseradish peroxidase-conjugated secondary antibodies and chemiluminescence reagents were from Pierce.

Plasmids (Fig. 1). A clone within the expressed sequence tag database (GenBank accession no. AA285249) containing the entire coding sequence of Chk2 was obtained from Thanos D. Halazonetis (Wistar Institute). For expression in mammalian cells, Chk2-coding sequences were amplified by PCR and cloned into the pcDNA3xHA-Neo and pcDNA3xFlag-Neo vectors, resulting in pcDNA-HAChk2 and pcDNA-FlagChk2, respectively. Point and internal deletion mutants were generated from pcDNA-HAChk2 by PCR-based site-directed mutagenesis (63).

For expression in *Escherichia coli*, Chk2 sequences (Fig. 1) were cloned into pGEX4T vectors (Amersham Pharmacia Biotech) for GST fusions and pTrcHis vectors (Invitrogen) for His-tagged fusions. Human Cdc25C (amino acids 200 to 256) and Cdc25A (amino acids 101 to 140) were isolated by PCR from expressed sequence tag clones (GenBank accession no. AW401554 and BE743496, respectively). Two copies of the FK506-binding protein (FKBP) mutant were isolated by PCR from pC4M-Fv2E (Ariad Pharmaceuticals, Inc., Cambridge, Mass.), digested with *Apo*I and *Eco*RI, and subcloned into the *Eco*RI site of the pcDNA3xHA and pcDNA3xFlag vectors. The resulting constructs were designated pcDNAHA2FKBP and pcDNAFlag2FKBP, respectively. Chk2 and its mutants were subcloned into these vectors.

Plasmid constructs were verified by sequence analysis. Primer sequences and detailed cloning strategies are available upon request. Wild-type and kinase-defective (D2870A and N2875K, respectively) Flag-*ATM* constructs (8) were kind gifts from Michael Kastan (St. Jude Children's Hospital). pGEX-Chk2(1-222) and pGEX-Chk2(57-222) (9) were obtained from Susan Lees-Miller (University of Calgary). pC4M-Fv2E, containing two copies of mutated FKBP, was provided by Ariad Pharmaceuticals, Inc. (Cambridge, Mass.).

Recombinant proteins. Expression of GST fusions or His fusions in *E. coli* strain DH5α was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 to 4 h at 37°C. For GST fusion proteins, cell lysates were harvested in PBS (Dulbecco's phosphate-buffered saline lacking Ca^{2+} and Mg^{2+}) in the presence of 1 mg of lysozyme and 10 U of DNase I per ml. For His fusion proteins, cell lysates were collected in 50 mM NaH_2PO_4–300 mM NaCl–10 mM imidazole, pH 8.0, in the presence of 1 mg of lysozyme and 10 U of DNase I per

ml. Cell lysates then went through 10 cycles of freezing and thawing. GST and His fusion proteins were batch purified with glutathione-Sepharose beads (Amersham Pharmacia Biotech) or Ni-nitrilotriacetic acid (NTA) beads (Qiagen), respectively, according to the manufacturers' procedures. GST fusion proteins were eluted with 50 mM Tris–10 mM glutathione, pH 8.0. His fusion proteins were eluted with 50 mM NaH_2PO_4–300 mM NaCl–250 mM imidazole, pH 8.0, and then dialyzed against PBS.

In vitro coupled transcription-translation assays. Chk2 constructs (pcDNA series) were used as templates for in vitro transcription-translation of Chk2 in the absence or presence of [^{35}S]Met-Cys labeling mix (Amersham Pharmacia Biotech). Promega TNT T7 quick coupled transcription-translation reticulocyte lysate system and T7 coupled transcription-translation wheat germ extract system were used in a standard 50-μl reaction according to procedures recommended by the manufacturer.

Cell culture and transfection. *ATM*-deficient (GM5849C) simian virus 40-transformed human fibroblasts were obtained from the Coriell Institute for Medical Research, Camden, N.J. Other cell lines were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U of penicillin/ml, and 50 mg of streptomycin/ml. Transfection was performed with Fugene 6 (Roche) at a ratio of 1 μg of plasmid to 2 μl of Fugene. Stable transfectants were selected in medium containing G418 (Life Technologies) at 700 μg/ml. Cells were treated with 1 mM hydroxyurea (Sigma) 24 h after transfection for 24 h. Cells were irradiated in a Mark I ^{137}Cs irradiator (Shepherd) at a dose rate of 1.8 Gy/min 48 h after transfection. Cells were UV irradiated at a dose of 50 J/m^2 with a Stratagene Cross-linker 48 h after transfection.

Immunoprecipitation and immunoblotting. Cell lysate was harvested 2 h after irradiation or 24 h after hydroxyurea treatment in TSD buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 0.1% sodium deoxycholate, 0.1% Triton X-100, and protease inhibitor cocktail [Roche]). Two micrograms of antibodies was used for immunoprecipitation from 400 to 500 μg of total lysate at 4°C overnight. Precipitates were washed with TSD buffer lacking protease inhibitors. In vitro translation product was mixed with 300 μl of NETN (20 mM Tris-HCl [pH 8.0], 0.1 M NaCl, 1 mM EDTA, 0.5% NP-40, and protease inhibitor cocktail) for immunoprecipitation. Precipitates were washed with NETN buffer lacking protease inhibitors. Immunoblots on nitrocellulose were blocked with 5% nonfat milk in PBST (PBS with 0.5% Tween 20) and washed in PBST.

GST pulldown experiments. Two micrograms of soluble GST fusion proteins and 20 μl of glutathione-Sepharose beads were incubated with 500 μg of total lysate in TSD buffer derived from HEK 293 cells expressing HA-tagged Chk2 and mutants (Fig. 6C, 7C, and 7D) or with 0.5 μg of soluble wild-type or kinase-defective His-Flag-Chk2 in the presence of 300 μl of NETN buffer (Fig. 6A, 6B, and 8) at 4°C overnight. The beads were washed in NETN buffer lacking protease inhibitors.

In vitro kinase assays. Kinases (prepared as soluble GST or His fusion proteins, immune complexes, or GST affinity isolates) were incubated with substrates at 30°C for 5 to 10 min in 1× kinase buffer (20 mM Tris [pH 7.5], 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol) supplemented with either 2 μM nonradioactive ATP or 2 μM nonlabeled ATP and 10 μCi of [γ-^{32}P]ATP (>5,000 Ci/mmol; AA0018, Amersham Pharmacia Biotech).

Phosphatase treatment. Immunoprecipitates of HA-Chk2 produced by translation in the coupled reticulocyte lysate system were incubated with calf intestinal phosphatase (New England Biolabs) for 1 h at 37°C. Soluble wild-type His-Flag-Chk2 (0.5 μg) was incubated with 1,000 U of λ phosphatase (New England Biolabs) in the presence of 2 mM $MnCl_2$ for 1 to 2 h at 30°C in a 50-μl reaction volume.

Induced oligomerization of Chk2 chimeras. HEK 293 cells were transiently transfected with pcDNAHA2FKBP-Chk2 (or its kinase-defective mutant) and/or pcDNAFlag2FKBP-Chk2 (or its kinase-defective mutant). Forty-eight hours later, transfectants were either mock-treated with 0.1% ethanol or treated with a 10 nM concentration of the bivalent ligand AP20187 (Ariad Pharmaceuticals, Inc.) for the FKBP mutant. Where indicated, transfectants were exposed to 10-Gy or 2.5-Gy gamma irradiation immediately before adding ligand. Lysates were harvested between 2 and 6 h after treatment. All solutions for immunoprecipitation, washing, and kinase assays for the AP20187-treated samples contained 10 μM AP20187.

RESULTS

Cell-free system for activation of Chk2. In *S. cerevisiae*, DNA damage induces a stable modification of the Chk2 homolog Rad53 that results in elevated activity detectable by in-gel kinase assays after denaturing gel electrophoresis and subsequent renaturation (45). This modification, probably phosphorylation, depends upon the Atm/Atr homolog Mec1. However, efforts to activate mammalian Chk2 in vitro by phosphorylation with Atm (41) or DNA-dependent protein kinase (DNA-PK) (X. Xu and D. F. Stern, unpublished data) have been unsuccessful.

Chk2 produced by coupled in vitro transcription-translation in rabbit reticulocyte lysates migrates heterogeneously in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Fig. 2A, lane 1). The slower-migrating form was eliminated by phosphatase treatment (Fig. 2A, lanes 3 and 4). Expression of the kinase-defective D347A allele in rabbit reticulocyte lysates yielded only the hypophosphorylated form (Fig. 2A, lane 2), indicating that autophosphorylation is required for the mobility shift. Phosphorylation of immunoreactive T26/S28 and T68 in the SCD was evident in both wild-type and kinase-defective Chk2 derived from rabbit reticulocyte lysates (Fig. 2B, lane 12). Thus, one or more protein kinases that can phosphorylate Chk2 at these sites are present in the rabbit reticulocyte lysates. Since the kinase-defective form does not undergo an extreme mobility shift (Fig. 2A, lane 2), phosphorylation at T26/S28 and/or T68 is not sufficient to retard the mobility of Chk2.

The simplest explanation for these results is that an endogenous Chk2 activating kinase is present in this system, with likely candidates being endogenous PIKKs and/or Chk2 (see below). Preincubation of rabbit reticulocyte lysates with 1 mM caffeine or 5 μM wortmannin inhibited phosphorylation at T26/S28 and at T68 (Fig. 2B, lanes 3 and 7). At these concentrations, caffeine inhibits human Atm and, partially, Atr but not DNA-PK, and wortmannin inhibits both Atm and DNA-PK but not Atr (48, 49). Loss of phosphorylation at these sites was accompanied by loss of Chk2 autophosphorylation activity, assayed by ^{32}P incorporation (Fig. 2B, lanes 3 and 7). We conclude that Chk2 derived from the rabbit reticulocyte lysates is phosphorylated by an Atm-like kinase and that this phosphorylation is required for strong Chk2 kinase activity.

In contrast to material produced in rabbit reticulocyte lysates, HA-Chk2 derived by translation in wheat germ extracts was not hyperphosphorylated, lacked T26/S28 and T68 phosphorylation, and had minimal autophosphorylation activity (Fig. 2B, lane 14, and Fig. 2C, lane 2). This system probably lacks an endogenous kinase that is capable of activating mammalian Chk2. Incubation of this material in rabbit reticulocyte lysates enhanced T68 phosphorylation and Chk2 autophosphorylation (Fig. 2C, lane 3; compare to Chk2 produced in rabbit reticulocyte lysates in lane 5). To our knowledge, this is the first mammalian cell-free system to enable catalytic activation of Chk2.

SCD is phosphorylated by Chk2. In the rabbit reticulocyte lysate system and in intact cells exposed to ionizing radiation, kinase-defective Chk2 is phosphorylated in *trans* at T26/S28 and T68 (Fig. 2B and data not shown). However, the full phosphorylation of Chk2 resulting in electrophoretic mobility shift requires a functional Chk2 kinase domain (Fig. 2B, lanes 2 and 12, and data not shown). This suggests that, at minimum, Chk2 electrophoretic mobility shift requires successive *trans* phosphorylation and autophosphorylation. Phosphorylation of Chk2 within the activation loop of the kinase domain is re-

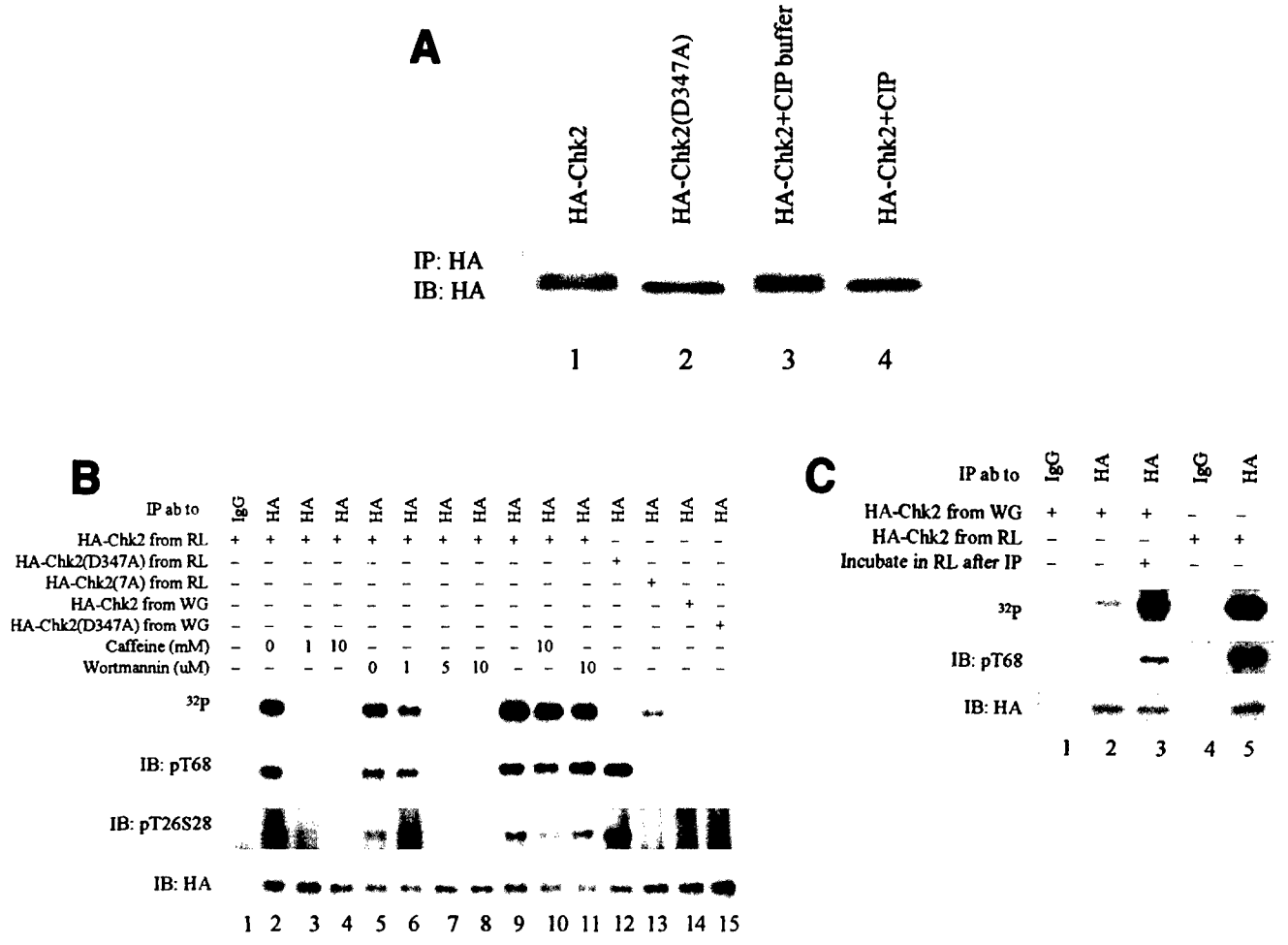


FIG. 2. Cell-free phosphorylation and activation of Chk2. HA-Chk2 or kinase-defective HA-Chk2(D347A) was produced by coupled transcription-translation in reticulocyte lysate (RL) or wheat germ lysate (WG) and isolated by immunoprecipitation with anti-HA. (A) Phosphorylation of HA-Chk2 produced in vitro. HA-Chk2 or HA-Chk2(D347A) produced in rabbit reticulocyte lysates was immunoprecipitated and detected by immunoblotting with anti-HA. For phosphatase experiments (right lanes), immunoprecipitated HA-Chk2 was incubated in calf intestinal phosphatase (CIP) buffer or calf intestinal phosphatase buffer plus calf intestinal phosphatase (1). HA-Chk2 produced in the reticulocyte lysate in vitro translation system is phosphorylated. Both HA-Chk2 and HA-Chk2(D347A) were in vitro transcribed-translated in the reticulocyte lysate with [^{35}S]Met-Cys for labeling and precipitated with anti-HA antibody. The immunoprecipitates of HA-Chk2 were treated with alkaline phosphatase buffer alone or plus alkaline phosphatase at 37°C for 1 h. IP, immunoprecipitation; IB, immunoblotting; ab, antibody. (B) Chk2 phosphorylation and kinase activity. Forms of HA-Chk2 produced by cell-free transcription-translation were immunoprecipitated and incubated with [γ-^{32}P]ATP for in vitro kinase assays. Recovery of Chk2 was monitored by immunoblotting with anti-HA (bottom). Phosphorylation at T68 or T26/S28 was measured by immunoblotting with the appropriate phosphospecific antibody; in vitro kinase activity was monitored by incorporation of [γ-^{32}P]ATP (top). The ^{32}P, phospho-T68, and HA panels are all from the same filter probed with anti-phospho-T68, stripped, and reprobed with anti-HA. Phospho-T26 (phospho-T68) and S28 are from an independent blot of portions of the same samples. In lanes 2 through 8, forms of HA-Chk2 were produced in the rabbit reticulocyte lysate system after incubation of the lysate with the designated concentration of vehicle control, caffeine, or wortmannin. Both caffeine and wortmannin inhibit Chk2 *trans*-phosphorylation and autophosphorylation. HA-Chk2 and its mutants were produced in either the in vitro translation wheat germ extract system or the in vitro translation reticulocyte lysate system, in which reticulocyte lysates were either mock treated or incubated with the indicated concentrations of caffeine or wortmannin. In lanes 9, 10, and 11, in vitro kinase assays were performed on the anti-HA immunoprecipitates in the presence of [γ-^{32}P]ATP only (lane 9) or in the presence of [γ-^{32}P]ATP and caffeine (10 mM, lane 10) or wortmannin (10 μM, lane 11) (top panel). IP, immunoprecipitation; IB, immunoblotting; ab, antibody. (C) Activation of Chk2 produced in wheat germ lysates. Phosphorylation of T68 and T26S28 was examined with Chk2 phospho-specific antibodies. HA-Chk2 was produced in the in vitro translation wheat germ extract system (lanes 1 to 3) or reticulocyte lysate system (lanes 4 and 5). HA-Chk2 was then precipitated with anti-HA antibody (lanes 2, 3, and 5) or mouse IgG (lanes 1 and 4). One Chk2 precipitate from the wheat germ extract system (lane 3) was incubated with reticulocyte lysate at 30°C for 30 min prior to the kinase assay. All the immunocomplexes were incubated with [γ-^{32}P]ATP for in vitro kinase assays. Recovery of Chk2 was monitored by immunoblotting with anti-HA (bottom panel) or with anti-phospho-T68 (middle panel) or by incorporation of [γ-^{32}P]ATP (top panel). IP, immunoprecipitation; IB, immunoblotting; ab, antibody.

quired for catalytic activation of Chk2 (32), and other autophosphorylation sites may also contribute to the mobility shift.

We analyzed a series of bacterially expressed GST-Chk2 alleles in order to identify domains in Chk2 that are required for kinase activity and domains that are subject to autophosphorylation (Fig. 3). In these assays, both autophosphorylation of Chk2 and *trans* phosphorylation of a GST-Cdc25C substrate peptide were monitored. Both of the kinase-defective alleles,

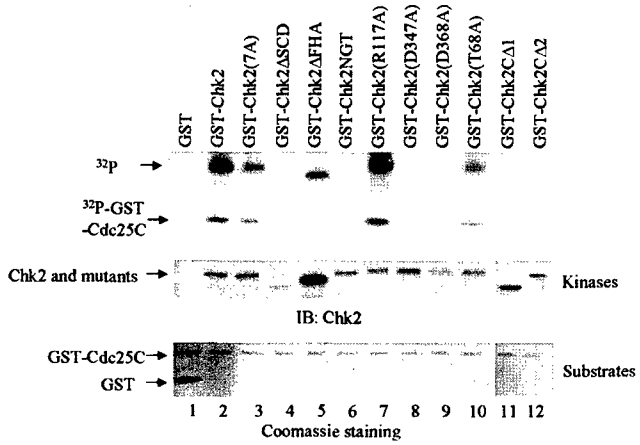


FIG. 3. Mutations in either SCD or FHA domain impair Chk2 kinase activities. GST fusion proteins were produced in bacteria, purified on glutathione beads, and released in soluble form with reduced glutathione. In vitro kinase assays were performed in the presence of Chk2 substrate GST-Cdc25C (amino acids 200 to 256) and [γ-^{32}P]ATP. Upper panel, [γ-^{32}P]ATP incorporation into GST-Chk2 and mutants; middle panel, [γ-^{32}P]ATP incorporation into GST-Cdc25C. Bottom panel, GST-Chk2 fusion proteins were quantified by immunoblotting with anti-Chk2 antibody (N-17). Coomassie brilliant blue staining showed substrate GST-Cdc25C. IB, immunoblotting.

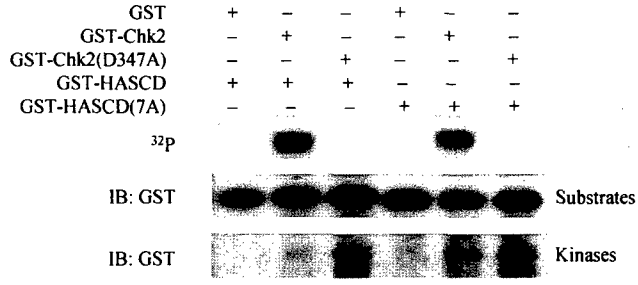


FIG. 4. Chk2 phosphorylates the SCD in vitro. In vitro kinase assays were performed on soluble GST-Chk2 and mutants in the presence of the substrate GST–HA-SCD or GST–HA-SCD(7A) and [γ−^{32}P]ATP. The top panel shows [γ-^{32}P]ATP incorporation into GST–HA-SCD or GST–HA-SCD(7A). GST–HA-SCD or GST–HA-SCD(7A) (middle panel). GST-Chk2 and its mutants (bottom panel) were detected by immunoblotting with anti-GST. IB, immunoblotting.

GST-Chk2(D347A) and GST-Chk2(D368A), lacked kinase activity in this assay. Within the SCD, the T68A substitution slightly reduced autophosphorylation and Cdc25C *trans*-phosphorylation activity. A more substantial decrease was observed with GST-Chk2(7A), in which all seven SQ/TQ sites within the SCD were replaced with AQ. Deletion of the entire SCD (GST-Chk2-ΔSCD) eliminated autophosphorylation, but only moderately diminished Cdc25 peptide phosphorylation. Similar results were observed with Chk2 and Chk2 mutants produced in the rabbit reticulocyte lysate system (data not shown). These data indicate that the SCD is required for maximal Chk2 kinase activity.

The stronger effects on autophosphorylation than on *trans* phosphorylation suggested that the SCD contains Chk2 autophosphorylation sites, including T68. Potential phosphorylation sites within the SCD include the seven SQ/TQ sites, as well as an additional 17 serines and 4 threonines. GST-Chk2 phosphorylated GST-SCD (Fig. 4, lane 3) and SCD(7A), lacking the SQ/TQ sites, to a lesser extent (Fig. 4, lane 5), but not GST (data not shown). A GST fusion protein of the Chk2 FHA domain was not phosphorylated (data not shown). Hence, the SCD is evidently a target for Chk2 autophosphorylation.

FHA domain is required for autophosphorylation of Chk2. FHA domains are phosphopeptide-binding modules (18, 19, 36, 37, 55, 57). Deletion of the core FHA domain of Chk2 resulted in a significantly lower autokinase activity and diminished *trans*-kinase activity (Fig. 3, lane 5). Similarly, triple mutation of the conserved FHA domain residues NGT (GST-Chk2-NGT) abrogated kinase activity (Fig. 3, lane 6). However, substitution of conserved R117 did not affect kinase activity. Similar results were obtained in the rabbit reticulocyte lysate system (data not shown). These data suggested that the FHA domain regulates Chk2 kinase activity, consistent with earlier work showing that mutations in the FHA domain prevent Chk2 activation in vivo (11, 32, 62).

Ionizing radiation enhances *ATM*-dependent oligomerization of Chk2. A yeast two-hybrid screen with yeast Rad53 as the bait identified Rad53 as an interacting protein (Z. Sun and D. F. Stern, unpublished results). Since this suggested that Rad53 forms dimers or oligomers, we determined whether Chk2 oligomers are produced in mammalian cells. In 293 cells expressing Flag-Chk2 and HA-Chk2, the tagged proteins coimmunoprecipitated, and the coimmunoprecipitation was enhanced by exposure to ionizing radiation (Fig. 5A and 5B). In *ATM*-deficient fibroblasts originating in a patient with ataxia-telangiectasia, enhancement of Chk2 oligomerization after gamma irradiation occurred with coexpression of wild-type Atm but not kinase-defective Atm (Fig. 5B). The higher baseline oligomerization compared to that in Fig. 5A probably reflects higher total expression of Chk2 with transient expression of both tagged proteins. These results demonstrated that at least two Chk2 molecules are components of an oligomer in vivo, that oligomerization is enhanced in response to DNA damage, and that this enhancement is *ATM* dependent. Thus, Chk2 oligomerization may be a regulated process that is linked to Chk2 activation.

Direct interactions of Chk2. Coimmunoprecipitation of two tagged Chk2 molecules may occur if they form homodimers or participate in a larger complex containing additional proteins. Bacterially expressed GST-Chk2 and kinase-defective GST-Chk2(D347A) interacted with His-Flag-Chk2 and His-Flag-Chk2(D347A) in GST pulldown experiments even though no other eukaryotic proteins were present (Fig. 6A and 6B). However, His-Flag-Chk2(D347A) does not interact well with GST-Chk2(D347A) (Fig. 6B). Hence, Chk2 homomers can form, provided that at least one molecule has catalytic activity.

We next determined if bacterially produced GST-Chk2 or kinase-defective GST-Chk2(D347A) would pull down HA-tagged Chk2 stably expressed in 293 cells. Both GST-Chk2 and GST-Chk2(D347A) preferentially bound to HA-Chk2 after gamma irradiation (Fig. 6C). Similar results were obtained with both GST pulldown and coimmunoprecipitation of wild-type and kinase-effective Chk2 in the rabbit reticulocyte lysate system (data not shown). In these experiments, with Chk2 produced in bacteria, rabbit reticulocyte lysates, or in mamma-

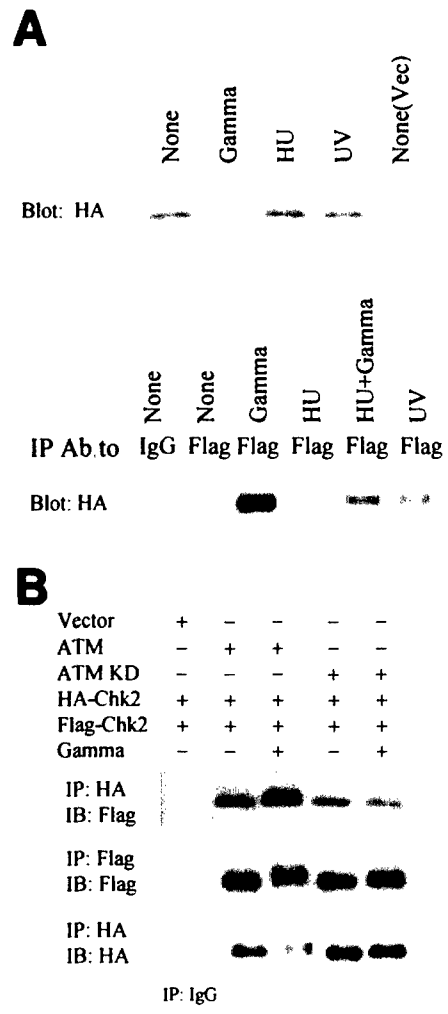


FIG. 5. Chk2 oligomerization. (A) 293 cells stably expressing Flag-Chk2 were transiently transfected with HA-Chk2. Transfectants were treated with 1 mM hydroxyurea for 24 h, beginning 24 h after transfection, or exposed to gamma irradiation (20 Gy) or UV (50 J/m^2) 48 h after transfection. Cell lysates were harvested 24 h after hydroxyurea treatment or 2 h after irradiation. Upper panel, expression and mobility shift of HA-Chk2 were determined by immunoblotting lysates with anti-HA antibody. Lower panel, coimmunoprecipitation with anti-Flag antibody was performed with equal portions of cell lysates and detected with anti-HA. (B) *ATM*-dependent increase in Chk2 coimmunoprecipitation. GM5849C ataxia-telangiectasia cells were transiently transfected with HA-Chk2 and Flag-Chk2, plus vector (pcDNA3), wild-type Flag-ATM, or kinase-defective Flag-ATMkd. Transfectants were exposed to 20 Gy of gamma irradiation 48 h after transfection. Cell lysates were harvested 2 h after irradiation. Equal amounts of lysates were immunoprecipitated with anti-HA (top and bottom panels) or anti-Flag (middle panel) antibodies. Precipitates were blotted for Flag-Chk2 (top two panels) or HA-Chk2 (bottom panel). IP, immunoprecipitation; IB, immunoblotting; Ab, antibody.

lian cells, kinase-defective Chk2 associated more strongly with Chk2 than did wild-type Chk2 (Fig. 6A and B and data not shown).

Chk2 oligomerization requires SCD and FHA domains. Domains required for Chk2 oligomerization were localized by mutational analysis. We first mapped the Chk2-interacting domain(s) by coimmunoprecipitation from 293 cells transiently expressing various forms of HA-tagged and Flag-tagged Chk2. We note that, at most, heterooligomers potentially represent only one-third of the total oligomerized Chk2 molecules (homooligomers of HA-tagged or Flag-tagged Chk2 or heterooligomers of HA-tagged and Flag-tagged Chk2). Deletion of the FHA domain virtually eliminated coimmunoprecipitation of Chk2 molecules.

Phosphorylation at T68 is induced by Atm and possibly Atr after DNA damage and is required for quantitative activation of Chk2 by ionizing radiation (1, 42, 64). Substitution of T68 with alanine only slightly reduced oligomerization both before and after gamma irradiation (Fig. 7A). Point substitution of other SQ/TQ sites (S19, T26/S28, S33/S35, and S50) did not significantly affect oligomerization (data not shown). However, mutation of all seven SQ/TQ sites, including T68, within the SCD significantly diminished coimmunoprecipitation (Fig. 7A). This suggests that Chk2 oligomerization involves one or more intact SQ/TQ sites within the SCD, but we cannot rule out a global structural effect of multiple substitutions. Deletion and substitution mutants of bacterially produced GST-Chk2 fusion proteins were used to determine the minimal fragment of Chk2 required for binding wild-type Chk2 produced in irradiated 293 cells (Fig. 7C). GST–HA-FHA was sufficient to preferentially bind wild-type Chk2 after DNA damage.

Since coimmunoprecipitation of two tagged Chk2 molecules required both the FHA domain and phosphorylation within the SCD, and since GST-FHA is sufficient to isolate Chk2 after DNA damage, we hypothesized that oligomerization of Chk2 is mediated by FHA/phospho-SCD interactions. Consistent with this hypothesis, GST–HA-FHA failed to pull down HA-Chk2ΔSCD in 293 cells after gamma irradiation. GST–HA-FHA did bind to HA-Chk2-SCD(7A) expressed in 293 cells after gamma irradiation (Fig. 7D), perhaps reflecting Chk2 autophosphorylation at non-SQ/TQ sites within the SCD (Fig. 4).

If an FHA domain in one Chk2 molecule associates with the phospho-SCD in another Chk2 molecule, then it should be possible to form a heterodimer containing one molecule with an intact phospho-SCD but deleted FHA domain, and another with a deleted SCD but intact FHA domain. Indeed, Flag-Chk2ΔSCD coimmunoprecipitated with HA-Chk2-ΔFHA, and the association was enhanced by ionizing radiation (Fig. 7B).

Chk2 oligomerization is phosphorylation dependent. In order to directly test the significance of phosphorylation on the putative FHA/phospho-SCD interaction, we determined whether the interaction is affected by phosphatase treatment. The ability of bacterially expressed GST-Chk2(D347A) to pull down bacterially produced His-Flag-Chk2 (Fig. 8A) was prevented by prior dephosphorylation of His-Flag-Chk2 with λ phosphatase (Fig. 8B). Similar results were obtained with the GST-FHA domain rather than full-length Chk2 as a pulldown reagent (Fig. 8B). In order to verify that this binding is mediated by the FHA domain rather than other sequences present in the fusion protein, we used GST fusion proteins containing and lacking the FHA domain and also GST1-221NGT, with alanine substitutions in a tripeptide important for FHA function. GST-Chk2 fusion proteins containing an intact FHA domain strongly bound to His-Flag-Chk2 but not His-Flag-Chk2(D347A). However, GST1-221NGT did not bind (Fig. 8C).

***trans* phosphorylation in the Chk2-Chk2 complex in vitro.** An important function of Chk2 oligomerization may be that it enables cross-phosphorylation of Chk2 molecules, which in

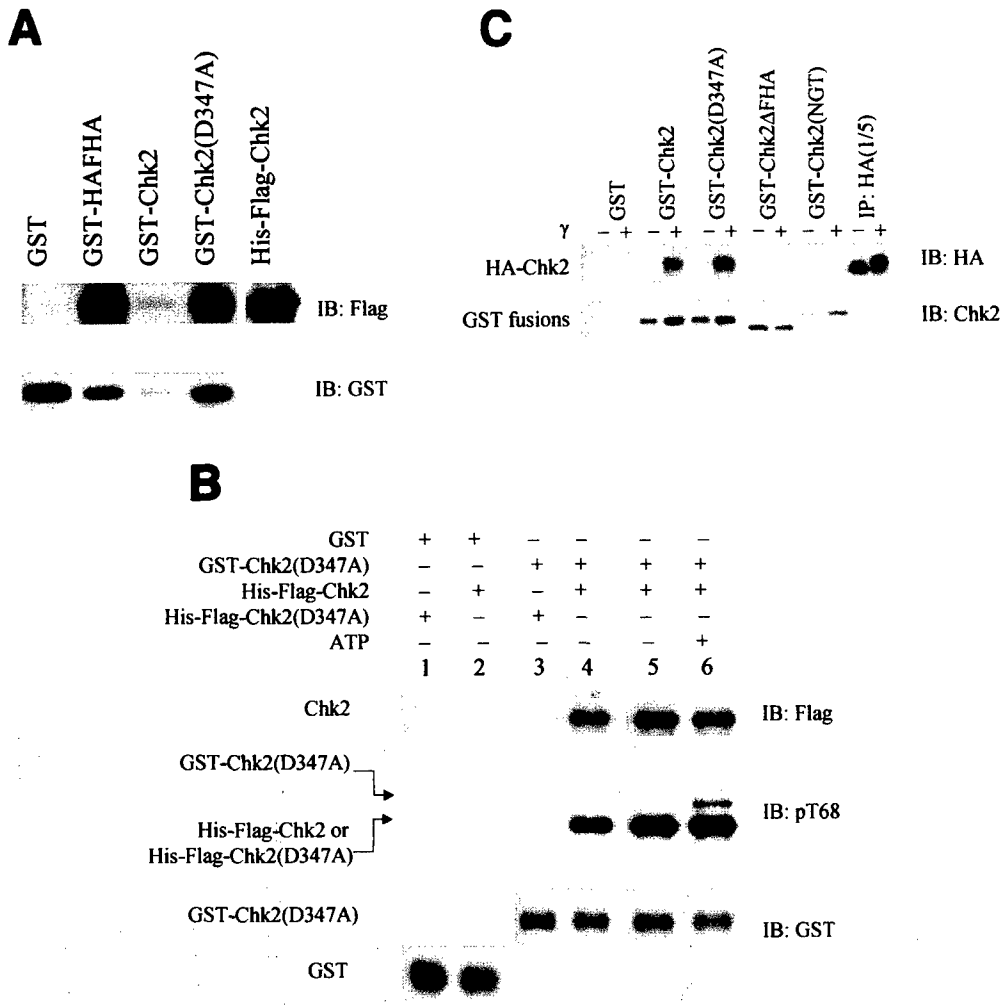


FIG. 6. Oligomerization of Chk2 produced in bacteria and 293 cells. (A) Bacterial expression. GST fusion protein pulldown experiment with bacterially produced soluble GST-HA-FHA, GST-Chk2, or kinase-defective GST-Chk2(D347A) incubated with His-Flag-Chk2. The pulldowns were blotted for wild-type His-Flag-Chk2 with anti-Flag antibody (top panel) and for total input of GST fusions with anti-GST antibodies (bottom panel). Images of different-sized GST fusion proteins in the bottom panel were cropped from one autoradiograph and aligned with one another to save space. (B) Phosphorylation of Chk2D347A by Chk2. Soluble GST-Chk2D347A produced in bacteria was used to pull down soluble His-Flag-Chk2 (lanes 4, 5, and 6) or kinase-defective His-Flag-Chk2(D347A) (lane 3). The affinity complexes were incubated in 1× kinase buffer in the absence (lane 5) or in the presence (lane 6) of ATP. Chk2 phosphorylation was evaluated by immunoblotting with anti-phospho-T68. GST-Chk2D347A is significantly larger than His-Flag-Chk2. (C) Mammalian expression. 293 cells stably expressing HA-Chk2 were exposed to 20 Gy of gamma irradiation. Cell lysates were harvested 2 h after irradiation. Lysates were normalized for protein concentration and used for pulldown with GST-Chk2 and its mutants. The GST pulldowns were blotted for HA-Chk2 with anti-HA antibody and for GST fusion protein with anti-Chk2 antibodies (N-17). Different sizes of GST fusions on the bottom panel were cropped and realigned from a single autoradiograph. IP, immunoprecipitation; IB, immunoblotting.

turn enhances Chk2 activation. Hence, we determined whether Chk2 could cross-phosphorylate a kinase-defective Chk2 molecule in a heterodimer. In vitro kinase assays with bacterially produced GST-Chk2(D347A) and His-Flag-Chk2 revealed that cross-phosphorylation of kinase-defective GST-Chk2(D347A) by His-Flag-Chk2 at least occurs at T68 (Fig. 6B).

Forced oligomerization of Chk2. We examined the functional consequences of Chk2 oligomerization in vivo by the regulated induction of dimerization. This system is based on the fact that the immunosuppressive drugs FK506 and rapamycin bind with high affinity to the cellular receptor FKBP12, which is an abundant, cytoplasmic 108-amino-acid protein (54). The synthetic ligand AP20187 binds with subnanomolar affinity to FKBPs with a single amino acid substitution, F36V (Fv), while binding with 1,000-fold lower affinity to the wild-type protein (14). Introduction of the FKBP (F36V) module into a heterologous protein allows ligand-dependent homo- and heterodimerization of the target proteins.

We introduced two tandem FKBP(F36V) modules into the amino terminus of wild-type or kinase-defective Chk2 (Fig. 1). When HEK 293 cells were cotransfected with both HA- and Flag-tagged 2FKBP-Chk2, approximately one-third of Flag-tagged 2FKBP-Chk2 coimmunoprecipitated with HA-tagged 2FKBP-Chk2 2 h after addition of the synthetic ligand (Fig. 9A). (Note that heterooligomerization between HA-tagged

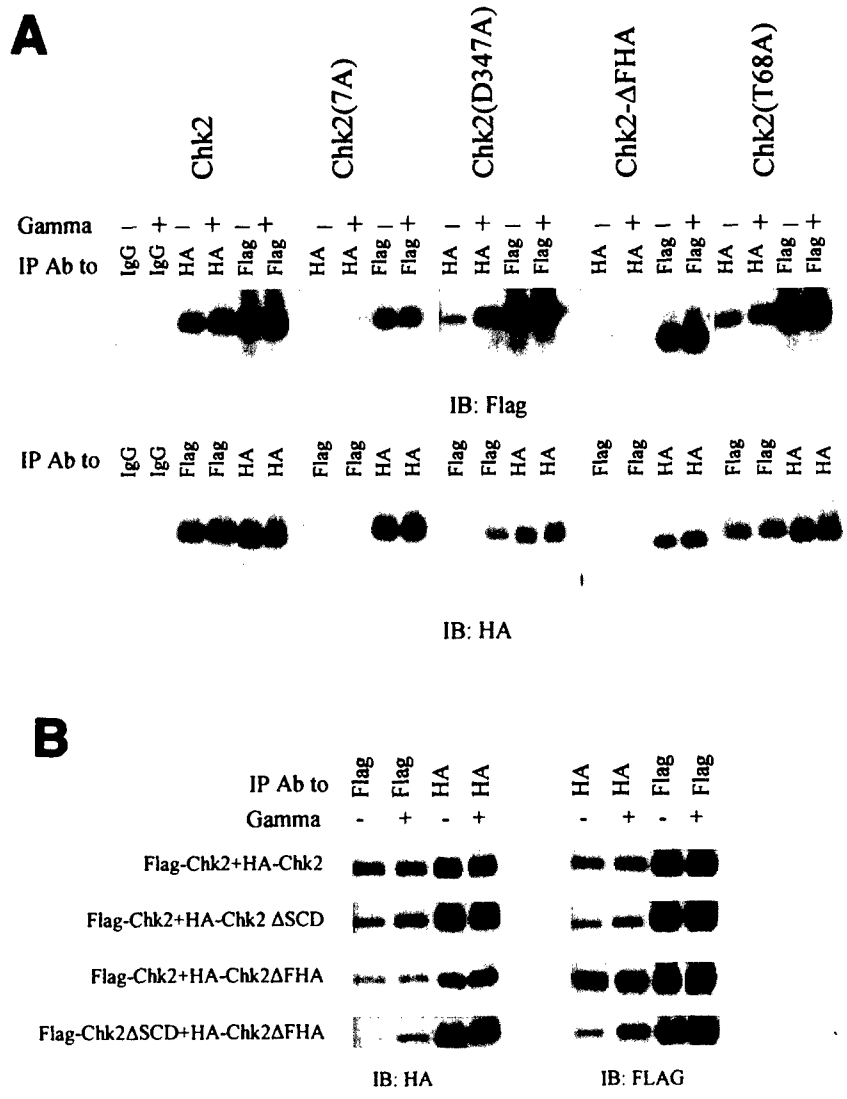


FIG. 7. Chk2 oligomerization domains. (A) Requirements for oligomerization in 293 cells. HA-tagged and Flag-tagged versions of individual Chk2 mutants were expressed by transient transfection in 293 cells. Transfectants were exposed to 20 Gy of gamma irradiation 48 h after transfection. Cell lysates were harvested 2 h after irradiation, and equal amounts were used for immunoprecipitation with anti-HA antibody or anti-Flag antibody as indicated and immunoblotted with anti-Flag (top panel) or anti-HA (bottom panel). Because homologous immunoprecipitations (e.g., IP anti-Flag, blot anti-Flag) were more efficient than heterologous coimmunoprecipitations (e.g., IP anti-Flag, blot anti-HA), only a one-fifth equivalent of homologous immunoprecipitation samples was analyzed relative to the cross-immunoprecipitation samples. (B) Oligomerization of FHA domain and SCD in vivo. Performed as in A except that Flag-tagged and HA-tagged Chk2 molecules were evaluated in the pairwise combinations listed at left. (C) GST-FHA binds to activated Chk2. Various Chk2-GST fusion proteins expressed in bacteria were used to isolate HA-Chk2 stably expressed in 293 cells. Experiments were performed as described in the legend to A. Equal portions of lysate from nonirradiated and irradiated cells were incubated with GST-Chk2 or its mutants. Pulldowns were blotted for HA-Chk2 with anti-HA antibody and for input of GST fusion protein with anti-GST antibodies. Different sizes of GST fusions on the bottom panel were cropped and realigned from one autoradiograph. Total lysate used for anti-HA immunoprecipitation was one-fifth of that for the GST pulldown. (D) Bacterially produced FHA domain of Chk2 binds to SCD in HA-Chk2 and its mutants expressed in 293 cells after gamma irradiation. The strategy described for Fig. 6C was used. Only one representative immunoblot for input of GST fusions (bottom panel) is shown. Different sizes of GST fusions on the bottom panel were cropped from one autoradiograph. (E) T68 phosphorylation of Chk2 and its mutants in vivo. Cells were handled essentially as described in A. Cell lysates were immunoprecipitated with anti-HA and detected by immunoblotting with anti-phospho-T68 or anti-HA. IP, immunoprecipitation; IB, immunoblotting; Ab, antibody.

and Flag-tagged 2FKBP-Chk2 potentially represents only one-third of the total amount of oligomerized 2FKBP-Chk2.) Ligand-induced oligomerization of Chk2 was significantly higher than that induced by ionizing irradiation (Fig. 9A). Induced oligomerization in vivo significantly increased T68 phosphorylation of wild-type Chk2 (Fig. 9B), reinforcing the conclusion that oligomerization facilitates Chk2 phosphorylation.

Surprisingly, kinase-defective Chk2 also showed greater T68 phosphorylation in the presence of ligand (Fig. 9B). Possible explanations include *trans* autophosphorylation in an oli-

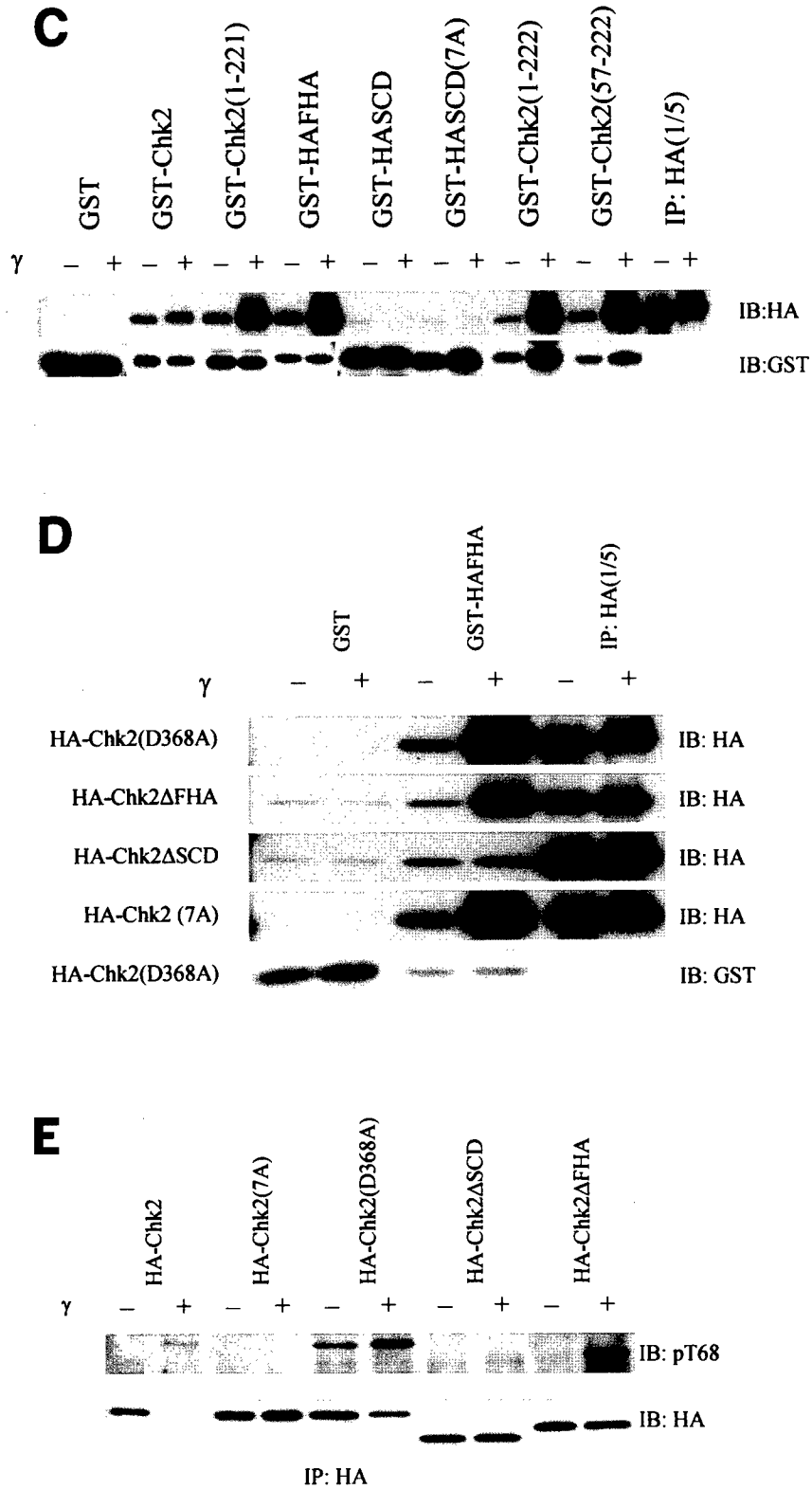
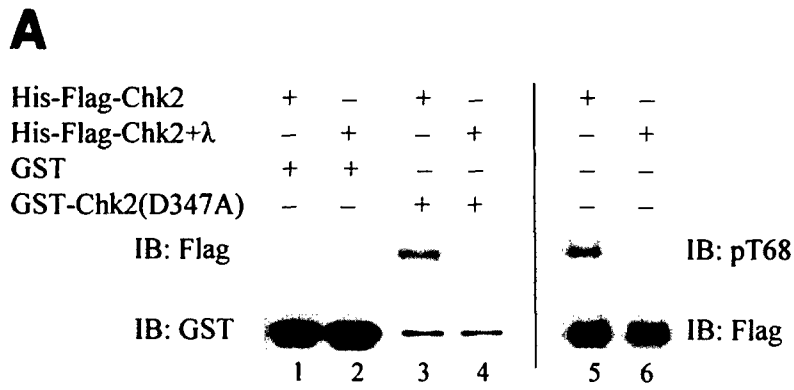
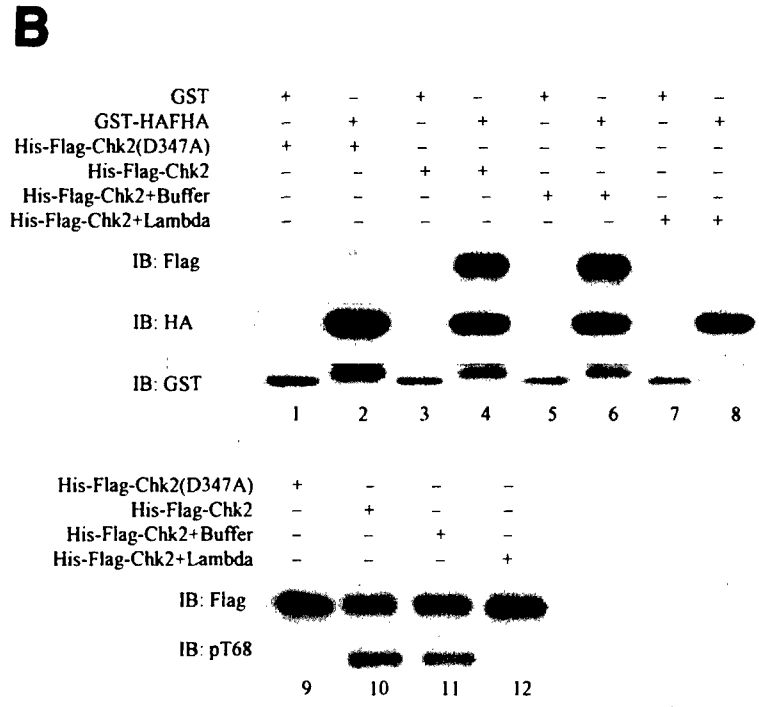
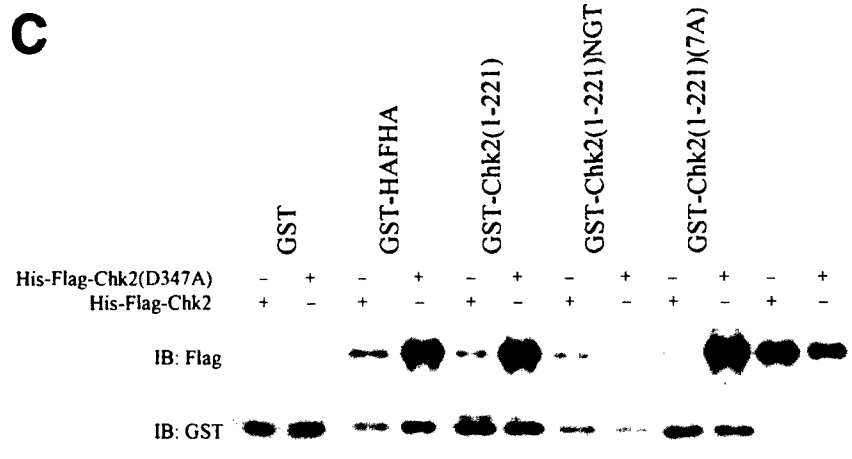


FIG. 7—*Continued.*

gomerized complex, in which kinase-defective Chk2 may associate with and be phosphorylated by the endogenous wild-type Chk2. Alternatively, the oligomerized Chk2 complex preferentially binds to and is phosphorylated by ATM/ATR kinases independent of Chk2 kinase activity. T68 phosphorylation in the ligand-induced oligomerized Chk2 immune complex was associated with increased Chk2 autokinase activity (Fig. 9C). However, this phosphorylation did not increase Chk2 *trans*


A
His-Flag-Chk2
His-Flag-Chk2+λ
GST
GST-Chk2(D347A)
IB: Flag
IB: GST
IB: pT68
IB: Flag
1 2 3 4 5 6


B
GST
GST-HAFHA
His-Flag-Chk2(D347A)
His-Flag-Chk2
His-Flag-Chk2+Buffer
His-Flag-Chk2+Lambda
IB: Flag
IB: HA
IB: GST
1 2 3 4 5 6 7 8
His-Flag-Chk2(D347A)
His-Flag-Chk2
His-Flag-Chk2+Buffer
His-Flag-Chk2+Lambda
IB: Flag
IB: pT68
9 10 11 12


C
GST
GST-HAFHA
GST-Chk2(1-221)
GST-Chk2(1-221)NGT
GST-Chk2(1-221)(7A)
His-Flag-Chk2(D347A)
His-Flag-Chk2
IB: Flag
IB: GST

phosphorylation of the Cdc25A polypeptide substrate (Fig. 9C), nor was it associated with measurable effects on cell cycle regulation or p53 stability (data not shown).

When Chk2 oligomerization was induced by ligand after exposure to a low dose of ionizing irradiation (2.5 Gy), both Chk2 auto- and *trans*-kinase activities increased (Fig. 9C) to levels comparable to that induced by 10-Gy irradiation (data not shown). This suggests that oligomerization plus a second DNA damage-dependent event, such as priming phosphorylation by ATM/ATR kinases, association with soluble activating or target proteins, or appropriate geometry of Chk2 oligomers induced by association with scaffolding molecules, is required for Chk2 activation and checkpoint pathway regulation.

DISCUSSION

We show here that Chk2 itself can phosphorylate Chk2 at T68 and other sites. Since T68 has already been identified as a *trans*-regulatory site, the Chk2-dependent phosphorylation of Chk2 has important implications for Chk2 regulation. Chk2 forms homomeric complexes in which the Chk2 FHA domain interacts with a second phosphorylated molecule of Chk2. Artificial dimerization of Chk2 in vivo concomitant with limited DNA damage augments Chk2 kinase activity. Finally, we provide evidence that Chk2 participates in DNA damage-dependent oligomeric complexes in vivo that have the same domain requirements as Chk2 homomers. These data suggest that the regulation of Chk2 by *trans* and autophosphorylation is more complicated than hitherto appreciated and involves a cascade of phosphorylation events that lead to the production of Chk2 homomeric complexes.

FHA domains are phosphopeptide interaction domains (18, 19, 36, 37, 55, 57). The Chk2 FHA domain is required for DNA damage-dependent Chk2 activation (11, 32, 62), and FHA domain mutations have been identified in alleles of Chk2 associated with variant $TP53^{+/+}$ Li-Fraumeni syndrome (5, 58) and a variety of tumors (27, 44). In the budding yeast DNA damage response, Rad53 FHA domains are required for DNA damage-dependent phosphorylation and activation of Rad53, which also requires the Atr homolog Mec1 and/or Tel1 (20, 47, 56, 57). A current model is that Mec1 is localized to sites of DNA damage and subsequently recruits and phosphorylates a second protein, Rad9, which is also required for damage-dependent activation of Rad53 (20, 47, 56, 57). Rad53 is recruited to the complex through interactions between Rad53 FHA domains and Rad9 phosphopeptides created by Mec1-mediated phosphorylation (M. F. Schwartz and D. F. Stern, submitted for publication). Since Mec1 is required for Rad53 activation and Rad53 has a cluster of potential PIKK phosphorylation sites similar to the Chk2 SCD, known to be a target for mammalian PIKKs, the relocalization of Rad53 mediated by FHA domains may be important for connecting Rad53 with the upstream activating PIKK Mec1. Another model, which is not incompatible with the first, is that dimerization of Rad53 through binding to dimeric Rad9 promotes Rad53 cross-phosphorylation and activation (22).

No mammalian Rad9 ortholog has been identified, nor has the binding partner for the Chk2 FHA domain. However, mutational analysis of Chk2 reveals the same dependencies of Chk2 function on the FHA domain (11, 32, 62), so that the same mechanisms probably operate in mammalian cells. With DNA damage, the initial function for the FHA domain will be coupling of Chk2 to upstream regulatory pathways, by bringing Chk2 to Atm/Atr and/or by adaptor- or scaffold-dependent activation. Our findings suggest that the FHA domain is required for an additional process involving intra- and/or intermolecular binding of the FHA domain to one or more phosphorylated sites within Chk2.

Similar to the Rad53 FHA domains, the Chk2 FHA domain is required for its PIKK-dependent phosphorylation. Point mutation (R145W) (33, 62) or deletion mutation (21 and 42 amino acids surrounding R145) (33) of the FHA domain abolished T68 phosphorylation. This suggests that the FHA domain couples Chk2 to an Atm- or Atr-activated complex. Nevertheless, we observed that deletion of the core FHA domain (amino acids 115 to 175) of Chk2 spared T68 phosphorylation after gamma irradiation (Fig. 7E) and binding to the bacterially produced FHA domain (Fig. 7D, second panel). This suggests that an additional mechanism of T68 phosphorylation bypassing the requirement for the Chk2 FHA domain can operate under some circumstances.

T68 is phosphorylated during damage-dependent activation of Chk2 in vivo and is likely to be a target for both Atm and Atr (1, 41, 42, 64). Phosphorylation at T68 is permissive for further Chk2 autophosphorylation at two sites in the activation loop, which is thought to be required for full activation of Chk2 (33). The mechanism by which T68 phosphorylation promotes Chk2 activation has not been determined. It may promote allosteric changes that are permissive for activation loop phosphorylation or may regulate intra- or intermolecular Chk2 interactions. Our finding that deletion of the FHA domain impairs the kinase activity of bacterially produced Chk2 suggests that this

FIG. 8. Phosphorylation-dependent oligomerization of Chk2. (A) Bacterially expressed His-Flag-Chk2 was incubated in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of λ phosphatase and either blotted directly with anti-phospho-T68 or anti-Flag (lanes 5 and 6) or pulled down with either GST or GST-Chk2(D347A) expressed in bacteria (lanes 1, 2, 3, and 4). Affinity-purified Chk2 was detected by blotting with anti-Flag, and the loading of GST proteins was monitored with anti-GST. (B) Phosphorylation-dependent binding of the Chk2 FHA domain. Bacterially expressed His-Flag-Chk2 or His-Flag-Chk2(D347A) was incubated in the absence (lanes 5, 6, and 11) or presence (lanes 7, 8, and 12) of λ phosphatase and immunoblotted with anti-Flag or anti-phospho-T68 (lanes 9, 10, 11, and 12). Bacterially produced GST or GST–HA-FHA was used to pull down additional portions of phosphatase-treated or nontreated kinase-defective His-Flag-Chk2(D347A) or His-Flag-Chk2. Wild-type and kinase-defective His-Flag-Chk2 were detected with anti-Flag antibody (upper panel), for input of GST–HA-FHA with anti-HA antibody (second panel) and for GST with anti-GST antibody (lower panel). (C) Effect of FHA domain mutations on binding to His-Flag-Chk2. GST fusion proteins expressed in bacteria were used to pull down bacterially produced wild-type and kinase-defective His-Flag-Chk2. His-Flag-tagged Chk2 was detected with anti-Flag antibody, and GST fusions were detected with anti-GST. Differently sized GST fusion proteins on the bottom panel were cropped and realigned from one autoradiograph. IP, immunoprecipitation; IB, immunoblotting.

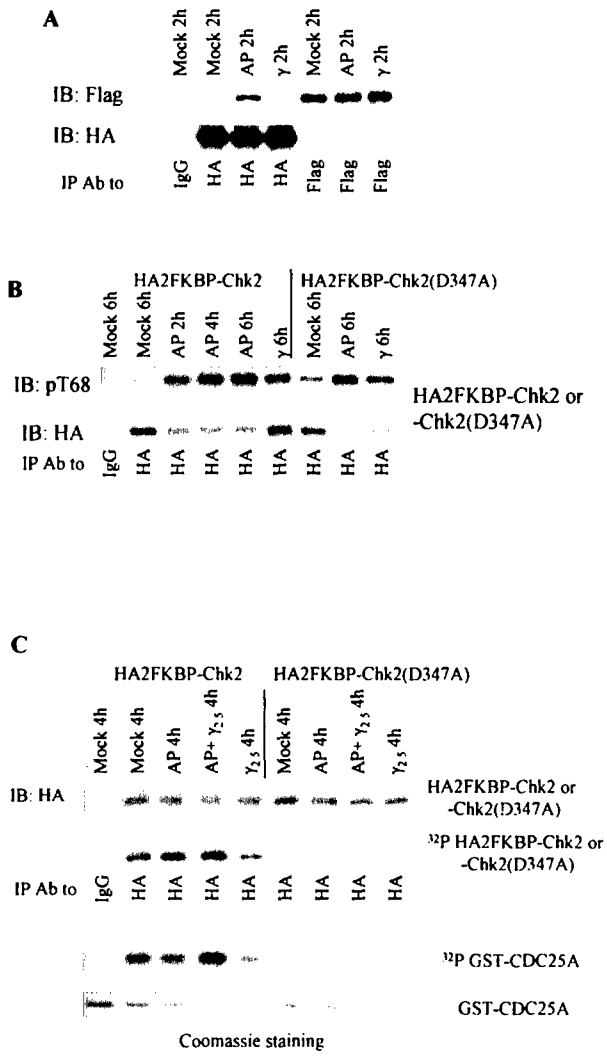


FIG. 9. Forced oligomerization and Chk2 activation. (A) AP20187 induced Chk2 oligomerization in vivo. HEK 293 cells were transiently cotransfected with both HA- and Flag-tagged 2FKBP-Chk2. Transfectants were either mock treated, treated with 10 μM AP20187, or irradiated with 10 Gy of gamma irradiation 48 h after transfection. Lysates were harvested 2 h after treatment and used for immunoprecipitation followed by immunoblotting. (B) Induced oligomerization resulted in T68 phosphorylation. Performed as in A, except that only HA-tagged wild-type or kinase-defective 2FKBP-Chk2 was transiently expressed. Lysates were harvested at each time point as indicated after treatment and used for immunoprecipitation with anti-HA antibody followed by immunoblotting for anti-phospho-T68 (top panel) or anti-HA (bottom panel, duplicate blot) antibodies. (C) Induced oligomerization after exposure to low-dose ionizing irradiation activated Chk2. Performed as in B, except that the dose of γ irradiation was 2.5 Gy and AP20187 was added to one set of 293 cells immediately after exposure to 2.5 Gy of irradiation. Immunocomplexes were incubated with [γ-^{32}P]ATP in the presence of bacterially produced GST-Cdc25A (amino acids 101 to 140). Recovery of Chk2 was monitored by immunoblotting with anti-HA (top panel) or incorporation of [γ-^{32}P]ATP (second panel). Recovery of GST-Cdc25A was detected by incorporation of [γ-^{32}P]ATP (third panel) or Coomassie staining (bottom panel). IP, immunoprecipitation; IB, immunoblotting; Ab, antibody.

domain has a positive regulatory influence. However, we cannot rule out the possibility that this is a result of nonspecific effects on Chk2 folding.

An important implication of the finding that Chk2 can phosphorylate itself at a known regulatory site is that phospho-Chk2 may be able to activate other molecules of Chk2. In this scenario, DNA damage-dependent activation of PIKKs would result in phosphorylation of Chk2 at T68 and other SQ/TQ sites within the SCD (1, 41, 42, 64). Phosphorylation of T68 would enable Chk2 autophosphorylation at the activation loop (32) and additional sites within the SCD. Nonphosphorylated Chk2 molecules would then be recruited and activated through FHA-SCD interactions and cross-phosphorylation. Hence, after priming phosphorylation by PIKKs, additional molecules could be activated independent of PIKK activity, thereby latching on Chk2 activation. This model is consistent with the intriguing finding that Rad9 can activate Rad53 in an ATP-dependent manner in the absence of Mec1 (22). Scaffolding of a Rad53 dimer by Rad9 could promote priming activation of Rad53, which could then activate additional molecules of Rad53. The catalytic function of Rad53 would be the source of the ATP dependence of the activation.

The ability of the Chk2 FHA domain to bind phospho-SCD suggests that FHA/phospho-SCD interactions may be important in intra- or intermolecular regulation of Chk2. Phosphorylation of the Chk2 SCD may enable an intramolecular interaction with the FHA domain that would either disturb a basal occupation of the kinase domain by the FHA domain or induce a structural change in the kinase domain, which is directly adjacent to the FHA domain. Alternatively, FHA/phospho-SCD interactions may enable recruitment of a second molecule of Chk2 through its FHA domain to a complex, thereby facilitating catalytic activation through cross-phosphorylation.

In other signaling systems involving phosphopeptide binding interactions, dynamic changes in association and localization of signaling proteins are mediated by a cascade of phosphopeptide/binding domain interactions. STATs are recruited to activated receptor tyrosine kinases by interaction of their SH2 domains with phosphopeptides on the phosphorylated receptors. Once recruited, the receptors phosphorylate the STATs, creating new STAT SH2 binding sites on the STATs themselves. An exchange of STAT-SH2/phosphoreceptor interactions with STAT-SH2/phospho-STAT interactions is an important step in releasing STATs from the sites of activation at the cell surface for transit to the nucleus, where they function as transcription factors (4, 13, 17).

Similarly, the R-Smad MH2 domain, a phosphoserine-binding motif structurally related to FHA domains, binds to phosphopeptides on the TGF-β-activated TGF-β type I receptor. Phosphorylation at the C-terminal serine residues of R-Smad by TGF-β type I receptor promotes homooligomerization by binding to the MH2 domain of the second R-Smad molecule and then dissociation from TGF-β type I receptor (46, 51, 61). It is noteworthy that unphosphorylatable co-Smad Smad4 competes with a phospho-R-Smad homooligomeric complex to form a more stable Smad4/phospho-R-Smad heterooligomeric complex (46, 61).

The ability of the Chk2 FHA domain to bind phospho-Chk2 as well as the likelihood that it binds to putative upstream activating proteins suggest a similar series of phosphopeptide binding site

exchanges in Chk2 activation. An overall model is that DNA checkpoint activation results in direct recruitment of PIKKs (ATR and ATM) and, independently, the Rad1-Rad9 (unrelated to budding yeast Rad9)-Hus1PCNA-related complex to sites of DNA damage (15, 31, 43, 66). DNA damage promotes relocalization of Chk2 phosphorylated at T68 (60). Recruitment of Chk2 would depend upon an interaction between its FHA domain and a phosphorylated constituent of the DNA damage complex. Once recruited, Chk2 would be activated by PIKK-dependent phosphorylation and/or by scaffolding-induced oligomerization. Additional Chk2 molecules would be activated similarly and by FHA domain-dependent binding of additional Chk2 molecules to already active and phosphorylated Chk2. Head-to-tail polymerization of phospho-Chk2 could potentially result in assembly of large active complexes.

Dissociation of Chk2 from the activated complexes could result from competition among FHA domain binding sites. We have found that phospho-Rad9 complexes with kinase-defective Rad53 are more abundant than those between phospho-Rad9 and kinase-active Rad53 (M. F. Schwartz and D. F. Stern, unpublished data). These data may be explained by a phosphorylation-dependent mechanism for dissociation of Chk2 FHA domain complexes that have already formed. If, hypothetically, the Chk2 FHA domain interacts more strongly with the Chk2 phospho-SCD than with the Rad9-like binding target in DNA damage complexes, then progressive activation of Chk2 would favor production of Chk2 homodimers over heteromers with the activation complex. This would result in release of active Chk2 homodimers, in much the same way that phosphorylated STATs and R-Smads are released from the activating receptors. Since heteromeric complexes between kinase-active Chk2 and inactive Chk2 are more readily isolated than those between two kinase-active molecules, even with bacterially expressed proteins, it is possible that the dual phosphorylated dimer is itself unstable and dissociates to release active monomers.

ACKNOWLEDGMENTS

We thank Michael Kastan and Susan Lees-Miller for plasmids and JoAnn Falato for secretarial assistance. We thank other members of the Stern laboratory for helpful comments, particularly Soo-Jung Lee, Jonathan McMenamin-Balano, and Marc F. Schwartz.

This work was supported by USAMRMC DAMD 17-98-1-8272, USPHS R01CA82257, and USAMRMC DAMD 17-01-1-0465 (X.X.), a Leslie H. Warner fellowship from the Yale Cancer Center (X.X.), an Anna Fuller fellowship in molecular oncology (L.T.), Susan G. Komen Breast Cancer Foundation fellowship PDF2000 719 (L.T.), and USAMRMC DAMD 17-01-1-0464 (L.T.).

ADDENDUM IN PROOF

Related work is reported by Ahn et al. (J. Y. Ahn, X. Li, H. L. Davis, and C. E. Canman, J. Biol. Chem., in press).

REFERENCES

1. **Ahn, J. Y., J. K. Schwarz, H. Piwnica-Worms, and C. E. Canman.** 2000. Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. Cancer Res. **60:**5934–5936.
2. **Anderson, C. W., and S. P. Lees-Miller.** 1992. The nuclear serine/threonine protein kinase DNA-PK. Crit. Rev. Eukaryot. Gene Expr. **2:**283–314.
3. **Banin, S., L. Moyal, S. Shieh, Y. Taya, C. W. Anderson, L. Chessa, N. I. Smorodinsky, C. Prives, Y. Reiss, Y. Shiloh, and Y. Ziv.** 1998. Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science **281:**1674–1677.
4. **Becker, S., B. Groner, and C. W. Muller.** 1998. Three-dimensional structure of the Stat3beta homodimer bound to DNA. Nature **394:**145–151.
5. **Bell, D. W., J. M. Varley, T. E. Szydlo, D. H. Kang, D. C. Wahrer, K. E. Shannon, M. Lubratovich, S. J. Verselis, K. J. Isselbacher, J. F. Fraumeni, J. M. Birch, F. P. Li, J. E. Garber, and D. A. Haber.** 1999. Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science **286:**2528–2531.
6. **Blasina, A., I. V. de Weyer, M. C. Laus, W. H. Luyten, A. E. Parker, and C. H. McGowan.** 1999. A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase. Curr. Biol. **9:**1–10.
7. **Brown, A. L., C. H. Lee, J. K. Schwarz, N. Mitiku, H. Piwnica-Worms, and J. H. Chung.** 1999. A human Cds1-related kinase that functions downstream of ATM protein in the cellular response to DNA damage. Proc. Natl. Acad. Sci. USA **96:**3745–3750.
8. **Canman, C. E., D. S. Lim, K. A. Cimprich, Y. Taya, K. Tamai, K. Sakaguchi, E. Appella, M. B. Kastan, and J. D. Siliciano.** 1998. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science **281:**1677–1679.
9. **Chan, D. W., S. C. Son, W. Block, R. Ye, K. K. Khanna, M. S. Wold, P. Douglas, A. A. Goodarzi, J. Pelley, Y. Taya, M. F. Lavin, and S. P. Lees-Miller.** 2000. Purification and characterization of ATM from human placenta. A manganese-dependent, wortmannin-sensitive serine/threonine protein kinase. J. Biol. Chem. **275:**7803–7810.
10. **Chaturvedi, P., W. K. Eng, Y. Zhu, M. R. Mattern, R. Mishra, M. R. Hurle, X. Zhang, R. S. Annan, Q. Lu, L. F. Faucette, G. F. Scott, X. Li, S. A. Carr, R. K. Johnson, J. D. Winkler, and B. B. Zhou.** 1999. Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway. Oncogene **18:**4047–4054.
11. **Chehab, N. H., A. Malikzay, M. Appel, and T. D. Halazonetis.** 2000. Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes Dev. **14:**278–288.
12. **Chehab, N. H., A. Malikzay, E. S. Stavridi, and T. D. Halazonetis.** 1999. Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. Proc. Natl. Acad. Sci. USA **96:**13777–13782.
13. **Chen, X., U. Vinkemeier, Y. Zhao, D. Jeruzalmi, J. E. Darnell, Jr., and J. Kuriyan.** 1998. Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA. Cell **93:**827–839.
14. **Clackson, T., W. Yang, L. W. Rozamus, M. Hatada, J. F. Amara, C. T. Rollins, L. F. Stevenson, S. R. Magari, S. A. Wood, N. L. Courage, X. Lu, F. Cerasoli, Jr., M. Gilman, and D. A. Holt.** 1998. Redesigning an FKBP-ligand interface to generate chemical dimerizers with novel specificity. Proc. Natl. Acad. Sci. USA **95:**10437–10442.
15. **Cortez, D., S. Guntuku, J. Qin, and S. J. Elledge.** 2001. ATR and ATRIP: partners in checkpoint signaling. Science **294:**1713–1716.
16. **Cortez, D., Y. Wang, J. Qin, and S. J. Elledge.** 1999. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. Science **286:**1162–1166.
17. **Darnell, J. E., Jr.** 1997. STATs and gene regulation. Science **277:**1630–1635.
18. **Durocher, D., J. Henckel, A. R. Fersht, and S. P. Jackson.** 1999. The FHA domain is a modular phosphopeptide recognition motif. Mol. Cell **4:**387–394.
19. **Durocher, D., I. A. Taylor, D. Sarbassova, L. F. Haire, S. L. Westcott, S. P. Jackson, S. J. Smerdon, and M. B. Yaffe.** 2000. The molecular basis of FHA domain:phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms. Mol. Cell **6:**1169–1182.
20. **Emili, A.** 1998. MEC1-dependent phosphorylation of Rad9p in response to DNA damage. Mol. Cell **2:**183–189.
21. **Falck, J., N. Mailand, R. G. Syljuasen, J. Bartek, and J. Lukas.** 2001. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature **410:**842–847.
22. **Gilbert, C. S., C. M. Green, and N. F. Lowndes.** 2001. Budding yeast Rad9 is an ATP-dependent Rad53 activating machine. Mol. Cell **8:**129–136.
23. **Hartwell, L. H., and M. B. Kastan.** 1994. Cell cycle control and cancer. Science **266:**1821–1828.
24. **Haruki, N., H. Saito, Y. Tatematsu, H. Konishi, T. Harano, A. Masuda, H. Osada, Y. Fujii, and T. Takahashi.** 2000. Histological type-selective, tumor-predominant expression of a novel CHK1 isoform and infrequent in vivo somatic CHK2 mutation in small cell lung cancer. Cancer Res. **60:**4689–4692.
25. **Hirao, A., Y. Y. Kong, S. Matsuoka, A. Wakeham, J. Ruland, H. Yoshida, D. Liu, S. J. Elledge, and T. W. Mak.** 2000. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science **287:**1824–1827.
26. **Hofmann, K., and P. Bucher.** 1995. The FHA domain: a putative nuclear signalling domain found in protein kinases and transcription factors. Trends Biochem. Sci. **20:**347–349.
27. **Hofmann, W. K., C. W. Miller, K. Tsukasaki, S. Tavor, T. Ikezoe, D. Hoelzer, S. Takeuchi, and H. P. Koeffler.** 2001. Mutation analysis of the DNA-damage checkpoint gene CHK2 in myelodysplastic syndromes and acute myeloid leukemias. Leukoc. Res. **25:**333–338.
28. **Inbal, B., G. Shani, O. Cohen, J. L. Kissil, and A. Kimchi.** 2000. Death-

associated protein kinase-related protein 1, a novel serine/threonine kinase involved in apoptosis. Mol. Cell. Biol. **20**:1044–1054.
29. **Khanna, K. K.** 2000. Cancer risk and the ATM gene: a continuing debate. J. Natl. Cancer Inst. **92**:795–802.
30. **Kim, S. T., D. S. Lim, C. E. Canman, and M. B. Kastan.** 1999. Substrate specificities and identification of putative substrates of ATM kinase family members. J. Biol. Chem. **274**:37538–37543.
31. **Kondo, T., T. Wakayama, T. Naiki, K. Matsumoto, and K. Sugimoto.** 2001. Recruitment of Mec1 and Ddc1 checkpoint proteins to double-strand breaks through distinct mechanisms. Science **294**:867–870.
32. **Lee, C. H., and J. H. Chung.** 2001. The hCds1 (Chk2)-FHA domain is essential for a chain of phosphorylation events on hCds1 that is induced by ionizing radiation. J. Biol. Chem. **276**:30537–30541.
33. **Lee, J. S., K. M. Collins, A. L. Brown, C. H. Lee, and J. H. Chung.** 2000. hCds1-mediated phosphorylation of BRCA1 regulates the DNA damage response. Nature **404**:201–204.
34. **Li, J., G. I. Lee, S. R. Van Doren, and J. C. Walker.** 2000. The FHA domain mediates phosphoprotein interactions. J. Cell Sci. **113**:4143–4149.
35. **Li, J., G. P. Smith, and J. C. Walker.** 1999. Kinase interaction domain of kinase-associated protein phosphatase, a phosphoprotein-binding domain. Proc. Natl. Acad. Sci. USA **96**:7821–7826.
36. **Liao, H., I. J. Byeon, and M. D. Tsai.** 1999. Structure and function of a new phosphopeptide-binding domain containing the FHA2 of Rad53. J. Mol. Biol. **294**:1041–1049.
37. **Liao, H., C. Yuan, M. I. Su, S. Yongkiettrakul, D. Qin, H. Li, I. J. Byeon, D. Pei, and M. D. Tsai.** 2000. Structure of the FHA1 domain of yeast Rad53 and identification of binding sites for both FHA1 and its target protein Rad9. J. Mol. Biol. **304**:941–951.
38. **Madhani, H. D.** 2001. Accounting for specificity in receptor tyrosine kinase signaling. Cell **106**:9–11.
39. **Massague, J., and Y. G. Chen.** 2000. Controlling TGF-beta signaling. Genes Dev. **14**:627–644.
40. **Matsuoka, S., M. Huang, and S. J. Elledge.** 1998. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science **282**:1893–1897.
41. **Matsuoka, S., G. Rotman, A. Ogawa, Y. Shiloh, K. Tamai, and S. J. Elledge.** 2000. Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. Proc. Natl. Acad. Sci. USA **97**:10389–10394.
42. **Melchionna, R., X. B. Chen, A. Blasina, and C. H. McGowan.** 2000. Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. Nat. Cell Biol. **2**:762–765.
43. **Melo, J. A., J. Cohen, and D. P. Toczyski.** 2001. Two checkpoint complexes are independently recruited to sites of DNA damage in vivo. Genes Dev. **15**:2809–2821.
44. **Miller, C. W., T. Ikezoe, U. Krug, W. K. Hofmann, S. Tavor, V. Vegesna, K. Tsukasaki, S. Takeuchi, and H. P. Koeffler.** 2002. Mutations of the CHK2 gene are found in some osteosarcomas, but are rare in breast, lung, and ovarian tumors. Genes Chromosomes Cancer **33**:17–21.
45. **Pellicioli, A., C. Lucca, G. Liberi, F. Marini, M. Lopes, P. Plevani, A. Romano, P. P. Di Fiore, and M. Foiani.** 1999. Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase. EMBO J. **18**:6561–6572.
46. **Qin, B. Y., B. M. Chacko, S. S. Lam, M. P. de Caestecker, J. J. Correia, and K. Lin.** 2001. Structural basis of Smad1 activation by receptor kinase phosphorylation. Mol. Cell **8**:1303–1312.
47. **Sanchez, Y., B. A. Desany, W. J. Jones, Q. Liu, B. Wang, and S. J. Elledge.** 1996. Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. Science **271**:357–360.
48. **Sarkaria, J. N., E. C. Busby, R. S. Tibbetts, P. Roos, Y. Taya, L. M. Karnitz, and R. T. Abraham.** 1999. Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res. **59**:4375–4382.
49. **Sarkaria, J. N., R. S. Tibbetts, E. C. Busby, A. P. Kennedy, D. E. Hill, and R. T. Abraham.** 1998. Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin. Cancer Res. **58**:4375–4382.
49a. **Schwartz, M. F., J. K. Duong, Z. Sun, J. S. Morrow, D. Pradhan, and D. F. Stern.** Rad9 phosphorylation sites couple Rad53 to the *Saccharomyces cerevisiae* DNA damage checkpoint. Mol. Cell, in press.
50. **Shani, G., S. Henis-Korenblit, G. Jona, O. Gileadi, M. Eisenstein, T. Ziv, A. Admon, and A. Kimchi.** 2001. Autophosphorylation restrains the apoptotic activity of DRP-1 kinase by controlling dimerization and calmodulin binding. EMBO J. **20**:1099–1113.
51. **Shi, Y.** 2001. Structural insights on Smad function in TGFbeta signaling. Bioessays **23**:223–232.
52. **Shieh, S. Y., J. Ahn, K. Tamai, Y. Taya, and C. Prives.** 2000. The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. Genes Dev. **14**:289–300.
53. **Sodha, N., R. Williams, J. Mangion, S. L. Bullock, M. R. Yuille, and R. A. Eeles.** 2000. Screening hCHK2 for mutations. Science **289**:359.
54. **Spencer, D. M., T. J. Wandless, S. L. Schreiber, and G. R. Crabtree.** 1993. Controlling signal transduction with synthetic ligands. Science **262**:1019–1024.
55. **Stone, J. M., M. A. Collinge, R. D. Smith, M. A. Horn, and J. C. Walker.** 1994. Interaction of a protein phosphatase with an *Arabidopsis* serine-threonine receptor kinase. Science **266**:793–795.
56. **Sun, Z., D. S. Fay, F. Marini, M. Foiani, and D. F. Stern.** 1996. Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways. Genes Dev. **10**:395–406.
57. **Sun, Z., J. Hsiao, D. S. Fay, and D. F. Stern.** 1998. Rad53 FHA domain associated with phosphorylated Rad9 in the DNA damage checkpoint. Science **281**:272–274.
58. **Vahteristo, P., A. Tamminen, P. Karvinen, H. Eerola, C. Eklund, L. A. Aaltonen, C. Blomqvist, K. Aittomaki, and H. Nevanlinna.** 2001. p53, CHK2, and CHK1 genes in Finnish families with Li-Fraumeni syndrome: further evidence of CHK2 in inherited cancer predisposition. Cancer Res. **61**:5718–5722.
59. **Vialard, J. E., C. S. Gilbert, C. M. Green, and N. F. Lowndes.** 1998. The budding yeast Rad9 checkpoint protein is subjected to Mec1/Tel1-dependent hyperphosphorylation and interacts with Rad53 after DNA damage. EMBO J. **17**:5679–5688.
60. **Ward, I. M., X. Wu, and J. Chen.** 2001. Threonine 68 of Chk2 is phosphorylated at sites of DNA strand breaks. J. Biol. Chem. **276**:47755–47758.
61. **Wu, J. W., M. Hu, J. Chai, J. Seoane, M. Huse, C. Li, D. J. Rigotti, S. Kyin, T. W. Muir, R. Fairman, J. Massague, and Y. Shi.** 2001. Crystal structure of a phosphorylated Smad2. Recognition of phosphoserine by the MH2 domain and insights on Smad function in TGF-beta signaling. Mol. Cell **8**:1277–1289.
62. **Wu, X., S. R. Webster, and J. Chen.** 2001. Characterization of tumor-associated Chk2 mutations. J. Biol. Chem. **276**:2971–2974.
63. **Xu, X., J. Liao, K. E. Creek, and L. Pirisi.** 1999. Human keratinocytes and tumor-derived cell lines express alternatively spliced forms of transforming growth factor-alpha mRNA, encoding precursors lacking carboxyl-terminal valine residues. Oncogene **18**:5554–5562.
64. **Zhou, B. B., P. Chaturvedi, K. Spring, S. P. Scott, R. A. Johanson, R. Mishra, M. R. Mattern, J. D. Winkler, and K. K. Khanna.** 2000. Caffeine abolishes the mammalian G_2/M DNA damage checkpoint by inhibiting ataxia-telangiectasia-mutated kinase activity. J. Biol. Chem. **275**:10342–10348.
65. **Zhou, B. B., and S. J. Elledge.** 2000. The DNA damage response: putting checkpoints in perspective. Nature **408**:433–439.
66. **Zou, L., D. Cortez, and S. J. Elledge.** 2002. Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. Genes Dev. **16**:198–208.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

ADB233865
ADB265530
ADB244706
ADB285843
ADB240902
ADB264038
ADB285885
ADB274458
ADB285735
ADB286597
ADB285707
ADB274521
ADB259955
ADB274793
ADB285914
ADB260288
ADB254419
ADB282347
ADB286860
ADB262052
ADB286348
ADB264839
ADB275123
ADB286590
ADB264002
ADB281670
ADB281622
ADB263720
ADB285876
ADB262660
ADB282191
ADB283518
ADB285797
ADB269339
ADB264584
ADB282777
ADB286185
ADB262261
ADB282896
ADB286247
ADB286127
ADB274629
ADB284370
ADB264652
ADB281790
ADB286578
ADB264750
ADB282776
ADB286264
ADB260563
ADB277918
ADB286365
ADB275327
ADB286736
ADB286137
ADB286146
ADB286100
ADB286266
ADB286308
ADB285832

ADB233865
ADB265530
ADB244706
ADB285843
ADB240902
ADB264038
ADB285885
ADB274458
ADB285735
ADB286597
ADB285707
ADB274521
ADB259955
ADB274793
ADB285914
ADB260288
ADB254419
ADB282347
ADB286860
ADB262052
ADB286348
ADB264839
ADB275123
ADB286590
ADB264002
ADB281670
ADB281622
ADB263720
ADB285876
ADB262660
ADB282191
ADB283518
ADB285797
ADB269339
ADB264584
ADB282777
ADB286185
ADB262261
ADB282896
ADB286247
ADB286127
ADB274629
ADB284370
ADB264652
ADB281790
ADB286578
ADB264750
ADB282776
ADB286264
ADB260563
ADB277918
ADB286365
ADB275327
ADB286736
ADB286137
ADB286146
ADB286100
ADB286266
ADB286308
ADB285832